THE DERMAL DELAYED TYPE HYPERSENSITIVITY REACTION IN SHEEP NATURALLY INFECTED WITH MAEDI-VISNA VIRUS

I.T.G. PYRAH

DOCTOR OF PHILOSOPHY
UNIVERSITY OF EDINBURGH
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DECLARATION

I hereby declare that this thesis was composed by myself, and describes work performed by myself except where specifically stated otherwise.

Mr. I.T.G. Pyrah, BVM&S, MRCVS

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ABSTRACT

Maedi Visna virus (MVV) is the prototype lentivirus, capable of infecting cells of the monocyte/macrophage series in sheep. Infection is associated with pathological changes characterised by dysfunction of the cell mediated immune system. The work described in this thesis was undertaken to study *in vivo* cell mediated immune function in sheep infected with MVV using the tuberculin driven delayed type hypersensitivity (DTH) reaction as the experimental system.

The gross and immunohistological characteristics of the DTH were firstly evaluated in control sheep ($CD4^+$, $CD8^+$, γδ, and B lymphocytes, macrophages and MHC class II expression). Grossly, the reaction consisted of an indurative plaque, maximal in size at 48-72 hours post challenge. Histologically, there was an early infiltrate of polymorphonuclear neutrophils (PMNs), with a subsequent influx of $CD4^+$ and $CD8^+$ T cells. Macrophages and MHC class II bearing cells left the lesion at the later stages. In comparison, MVV infected sheep exhibited a reduction in the size of the gross DTH which was significantly associated with a decreased density of PMNs and $CD4^+$ cells in the early reaction, but not with the degree of classical pathological change evaluated at subsequent post mortem or the presence of viral RNA in the skin.

Depletion of circulating PMNs using cytotoxic drugs in control sheep resulted in the depression in the size of the DTH and a reduced influx of $CD4^+$ cells, confirming the importance of PMNs in the development of the DTH lesion.

The migratory ability of PMNs and $CD4^+$ cells to the sites of dermally injected proinflammatory mediators (IL-8, TNF-α, and zymosan activated plasma) was subsequently shown to similar in control and MVV infected sheep.

Evaluation of the levels of circulating anti-PPD antibodies in the MVV infected and control groups provided evidence for a negative association between antibody levels and DTH size suggesting a switch to a Th2 type response in the MVV infected sheep.

Finally, the presence of cytokine mRNA (IL-10, TNF-α, IFN-γ and IL2-R) in the early lesions of both groups was assessed using reverse transcription polymerase chain reaction (RT-PCR) technology. This indicated that the mRNA expression pattern of these cytokines was not significantly different in control and MVV infected sheep.

In conclusion, the work has shown a significant depression of the DTH response in sheep infected with MVV. This depression is associated with decreased density of PMNs and $CD4^+$ cells in the early reaction, but is not associated with abnormalities in the trafficking of these cells to dermally injected proinflammatory mediators, the presence of antibody/antigen complexes, the presence of viral RNA, or difference in the cytokine mRNA production profile. The importance of the PMN in the DTH has been indicated, and a defect in the *in vivo* immune response of the PMN in MVV infected sheep has been described.

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ABBREVIATIONS

AEC	3-amino-9-ethylcarbazole
AGIDT	Agar gel immunodiffusion test
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
BALF	Bronchoalveolar lavage fluid
BCG	Bacillus Calmette Guérin
BIV	Bovine immunodeficiency virus
CAEV	Caprine arthritis encephalitis virus
cDNA	Copy DNA
CSF	Cerebrospinal fluid
ds	Double stranded
DTH	Delayed type hypersensitivity
EIAV	Equine infectious anaemia virus
ELAM-1	Endothelial adhesion molecule 1
ELISA	Enzyme linked immunosorbent assay
FIV	Feline immunodeficiency virus
HIV	Human immunodeficiency virus
IFN-γ	Interferon-γ
IL	Interleukin
LFA-1	Lymphocyte function antigen-1
LTR	Long terminal repeat
MHC	Major histocompatability complex
mRNA	Messenger RNA
MVV	Maedi-visna virus
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PMN	Polymorphonuclear neutrophil
PNK	Polynucleotide kinase
PPD	Purified protein derivative
rh	Recombinant human
ro	Recombinant ovine
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SIV	Simian immunodeficiency virus
SPA	Sheep pulmonary adenomatosis
ss	Single stranded
TNF-α	Tumour necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
ZAP	Zymosan activated plasma

CHAPTER ONE

MAEDI-VISNA VIRUS

1.1 GENERAL INTRODUCTION

Maedi-visna describes a geographically widespread ovine disease of viral aetiology. Infection is characterised by chronic, progressive lymphoid inflammatory lesions primarily affecting lung tissue, but also occurring in central nervous tissue, mammary gland tissue and synovial membranes. The clinical course of the disease is prolonged, with a gradual increase in the severity of clinical signs, and commonly results in the demise of the animal.

1.2 THE VIRUS

1.2.1 Classification

Maedi-Visna virus (MVV) is the a member of the *Retroviridae* family of viruses, this family being characterised by the presence of a positive sense single stranded RNA genome and the utilisation of a reverse transcriptase enzyme (Lin, Thormar, 1970). It is further classified into the *Lentivirinae* subfamily, so named as members of this family are characterised by a slow, yet predictable, disease progression (Sigurdsson, 1954a). Other members of this subfamily are capable of producing clinical disease in a range of animal species; bovine immunodeficiency virus (BIV) (Van Der Maaten et al. 1972; Gonda et al. 1987), caprine arthritis-encephalitis virus (CAEV) (Crawford et al. 1980), equine infectious anaemia virus (EIAV) (Kobayashi, 1961), feline immunodeficiency virus (FIV) (Pedersen et al.

1987), human immunodeficiency virus (HIV) (Barre-Sinoussi et al. 1983), and simian immunodeficiency virus (SIV) (Daniel et al. 1985).

1.2.2 Structure and replication

Electron microscopic studies have shown the infective virion to consist of a spherical or elliptical outer membrane of diameter of 65-110μm, with numerous small projecting spikes and an electron dense nucleoid (Thormar, 1961; Thormar, Cruickshank, 1965; Coward et al. 1970). The genome is composed of a 9-10 kilobase positive sense single stranded (ss) RNA (Brahic et al. 1977; Sonigo et al. 1985; Carey et al. 1993). At each end of the genome are replicated base sequences known as long terminal repeats (LTRs) which are involved in the control of viral replication. The majority of the remainder consists of three major gene regions *gag, pol,* and *env,* which code for core proteins, reverse transcriptase, integrase and protease enzymes, and envelope glycoprotein, respectively. The genome includes several short, multiply spliced open reading frames (ORFs), of which *tat, rev,* and *vif* genes have been characterised. These genes are considered to be involved in the control of replicative events (Carey et al. 1993).

The viral replicative cycle is initiated by the binding of the virus to a cell surface receptor molecule, putatively major histocompatability complex (MHC) class II antigen (Dalziel et al. 1991). This is followed by fusion of the virus with the cell membrane, which precedes viral entry and uncoating (Carey et al. 1993). The viral reverse transcriptase subsequently converts the viral ssRNA into proviral double stranded (ds) DNA (Haase, Varmus, 1973). This dsDNA then passes to the nucleus where it is present predominantly in a linear form, but also exists in a circular form

(Harris et al. 1981), and has been shown to integrate into the host genome via the action of a viral integrase encoded in the *pol* region of the viral genome (Clements et al. 1979; Katzman, Sudol, 1994). Integration into the host genome has not been proven to be a necessary stage in the viral life cycle (Carey et al. 1993). In vivo, there is control of viral protein expression at this stage of infection, with suppressed translation of this dsDNA into viral messenger RNA (mRNA) (Brahic et al. 1981). This contrasts with the in vitro cell culture models in which translation occurs rapidly and is followed by cell lysis and death (Thormar, 1961).

Translation of the *gag* and *pol* gene regions produces a 150 kDa precursor protein which cleaves to generate the reverse transcriptase and *gag* related proteins (Vigne et al. 1982).

The translation of the *gag* gene produces a precursor protein of 55kDa that is cleaved intracellularly to produce the three major internal structural proteins p30, p16 and p14 (Vigne et al. 1982).

Translation of the *env* component gives rise to the gp135 envelope glycoprotein and the gp46 transmembrane glycoprotein (Sonigo et al. 1985).

Translation of the characterised small ORFs leads to the production of the 10 kDa transactivator of viral transcription (*tat*) (Hess et al. 1985), a 19 kDa regulator of expression of viral protein (*rev*) product connected with *env* expression (Tiley et al. 1990), and a 29kDa *vif* protein of uncertain function (Audoly et al. 1992).

The ds DNA also acts as a template for the production of the viral ss RNA genome.

The mechanism of final viral particle assembly is not fully characterised, but is associated with budding from the cell membrane (Thormar, 1961).

The control of the life cycle has largely been studied in vitro, with evidence that there is a controlled sequence of transcription with smaller mRNAs expressed within 24 hours post innoculation, followed by larger molecules at the 72 hour time point (Sargan et al. 1994). The initial RNAs are postulated to code for the *tat* and *rev* messages (Gourdou et al. 1989; Tiley et al. 1990). *Tat* acts both as an activator of viral transcription via interaction with the LTR region (Neuveut et al. 1993), and has been found to have direct pathogenic effects in rats and mice, with neurotoxicity (Hayman et al. 1993) and multiorgan lymphoid hyperplasia (Vellutini et al. 1994) having been reported. *Rev* is required for the productive infection of cells in culture (Toohey, Haase, 1994), possibly acting to stabilise mRNA transcripts (Vigne et al. 1990). The combined action of these two factors facilitates active replication.

The relatively restricted level of replication occurring in vivo is not fully understood, although the upregulation of virus replication with cell activation (Gendelman et al. 1986) suggests the interaction of cellular factors with the virus control region can exert regulatory effects on virus multiplication (Carey et al. 1993).

1.2.3 Viral tropism

The virus is infective to both sheep and goats (Banks et al. 1983), with no evidence of other species acting as hosts (Carey et al. 1993).There is a suggestion of a variable susceptibility to infection between different breeds of sheep (Houwers et al. 1989).

Within the host, cells of the macrophage/monocyte series appear the most important target for the virus (Gorrell et al. 1992; Lujan et al. 1994), with the bone marrow monocyte precursor cell possibly acting as a reservoir of latent infection (Gendelman et al. 1985). Within the macrophage population, there is a selective infection with macrophages of the lung, lymph node, synovium, mammary gland, bone marrow, spinal cord, and spleen being particularly prone to infection(Kennedy et al. 1985; Gendelman et al. 1985; Brodie et al. 1992; Brodie et al. 1995). This phenomenon is possibly associated with the increased susceptibility to infection with increased activational status of the cell (Gendelman et al. 1986). Viral RNA has been located in macrophages, macrophage like cells and epithelium in vivo, although the productive infection was found to be restricted to cells of the macrophage series, with expression of viral proteins being further restricted to macrophages in the tissues affected with pathological changes (Brodie et al. 1995). There has been a single report of the productive infection of blood vessel smooth muscle cells in an *in vitro* study (Leroux et al. 1995).

1.2.4 Relationship with other members of the subfamily

Maedi visna virus is closely related to the other members of the *Lentivirinae* family genetically (particularly in the *gag* and *pol* regions of the genome), morphologically, and antigenically (Gonda et al. 1985; Stephens et al. 1986; Gonda et al. 1986; Maslak, Schmerr, 1993; Gonda et al. 1994).

1.3 CLINICOPATHOLOGICAL ASPECTS OF THE DISEASE

1.3.1 Historical perspective and occurrence

Maedi-visna, although previously reported (Marsh, 1923), first became prominent due to a severe epidemic of the disease in Iceland in the 1930s (Petursson, 1994). The disease was imported in a clinically inapparent form with Karakul sheep imported from Germany, and was disseminated in an alarming fashion largely due to the local sheep farming practices and the susceptibility of the local breed of sheep (Petursson, 1994). Subsequently the MVV has been recorded infecting a considerable proportion of the sheep population in many areas of the world (Rajya, Singh, 1964; Ressang et al. 1968; Wandera, 1970; Suveges, Szeky, 1973; Gates et al. 1978; Lujan et al. 1993; Bouljihad, Leipold, 1994)

The disease was first recognised serologically in the U.K. in 1979 (Dawson et al. 1979), with the first pathologically confirmed case in Scotland in 1981 (Grimshaw et al. 1981). A recent random serological survey of sheep flocks in England indicated that 1.6% of flocks sampled were infected, although the author considered this to be a gross underestimate when considering the limited percentage of the flock tested (Dawson, 1994).

1.3.2 Epidemiology

There is conflicting evidence as to the main pathway of transmission of MVV, although there appears to be a consensus that lambs born to seropositive ewes are more likely to seroconvert. Several authors have confirmed the prenatal infection of

lambs in utero, indicating a vertical transmission of the virus, with speculated mechanisms including viral genome integration into the germ cell line or the passage of somatic cells across the placenta (Cross et al. 1975; Cutlip et al. 1981; Brodie et al. 1994). This evidence has been challenged by other workers (De Boer et al. 1979; Houwers et al. 1987) who concluded that there was limited evidence of vertical transmission, and postulated that the epidemiologically significant method of transmission was horizontal, either via suckling and ingesting infected macrophages from the ewe's colostrum or inhalation of infected macrophages shed in respiratory secretions. It is apparent that close contact is required for infection to spread between individuals.

There is evidence that the coinfection of sheep with sheep pulmonary adenomatosis (SPA) increases the likelihood of the horizontal transmission of MVV (Pritchard, Done, 1990; Gonzalez et al. 1993).

1.3.3 Clinical presentation

MVV infection is associated with a range of clinical presentations, which may occur independently or in conjunction with each other, consisting of exercise intolerance associated with pulmonary disease (Maedi), progressive limb paresis and ataxia or vestibular disease (Visna), diffuse indurative mastitis, and swelling and crepitus associated with arthritis primarily affecting both carpal joints (Dawson, 1980; Oliver et al. 1981; Cutlip et al. 1985; van der Molen et al. 1985; Watt et al. 1992a; DeMartini et al. 1993; Brodie et al. 1995). Clinical evidence of infection only becomes apparent several years after infection, and as such sheep are generally 3 to 4 years of age before the onset of clinical symptoms (Sigurdsson, 1954b; Watt et al.

1992a). All sheep clinically affected by MVV exhibit wasting in the latter stages of the disease, but this is particularly evident when the disease sydrome Visna is apparent, 'visna' being the Icelandic term for wasting.

Sheep may survive for a period of months to years after the onset of clinical signs (Watt et al. 1992a).

Within the U.K., the respiratory form or Maedi is prevalent (Dawson, 1980; Watt et al. 1992a).

1.3.4 Pathology

Pathologically, MVV infection is characterised by a diffuse lymphocytic infiltrate in affected organs.

The affected lung fails to collapse, is heavier than normal, uniformly enlarged, firm and rubbery in consistency, with small multifocal grey spots on cut surfaces. The associated pulmonary lymph nodes are enlarged and exhibit hyperplasia. Histologically, there is diffuse interstitial infiltration with lymphocytes and monocyte/macrophages, a smooth muscle hyperplasia, and a predominantly lympocytic alveolitis. Lymphocytic germinal centre type structures are also diffusely present in the lung of many cases (Cutlip et al. 1979; Oliver et al. 1981; Lujan et al. 1991; Watt et al. 1992a; DeMartini et al. 1993; Lujan et al. 1993).The infiltrating lymphocytes present in the lung interstitium are of the $CD4^+$and $CD8^+$ phenotype, and have been found to be in the same ratio as control lung tissue (Watt et al. 1992b). The alveolitis has been characterised as having an increased number of $CD8^+$

lymphocytes (Cordier et al. 1992; Lujan et al. 1993), with a decreased number of $CD4^+$ lymphocytes being recorded by one group (Lujan et al. 1993) with a resultant inversion of the $CD4^+/CD8^+$ ratio. Expression of MHC class II has been found to be increased (Cordier et al. 1992; Lujan et al. 1993). The number of polymorphonuclear neutrophils (PMNs) is also raised (Cordier et al. 1992; Lujan et al. 1993). The hyperplastic regional lymphoid tissue exhibits an increase in the levels of $CD8^+$ and $\gamma\delta$ T cells in the follicles of the cortex (Watt et al. 1992b).

Affected central nervous tissue (CNS) is usually grossly normal, although there is a report of reddish streaking in the brain and spinal cord in some animals (Watt et al. 1992b). The typical histological changes consist of lymphocytic meningitis, lymphocytic infiltration of the neuroparenchyma with perivascular and choroid plexus lymphocyte/macrophage aggregations. There is evidence of demyelination, gliosis and astrocytic hypertrophy (Cutlip et al. 1979; Oliver et al. 1981; Watt et al. 1992b). The lymphocyte subsets involved in the CNS inflammation vary dependent upon the site examined with the perivascular infiltrates being predominantly $CD4^+$ and the diffuse neuroparenchymal response being predominantly $CD8^+$ (Torsteinsdottir et al. 1992). The latter results in a reversed $CD4^+/CD8^+$ ratio in this area. Many of these cells express MHC class II antigen (Torsteinsdottir et al. 1992).

Affected mammary gland is small and firm to cut, exhibiting a diffuse lymphocytic infiltration and fibrosis, with germinal centre type structures being found particularly periductally (Oliver et al. 1981; van der Molen et al. 1985; Lujan et al. 1991; Watt et al. 1992b).

Finally, the affected joints exhibit swollen, oedematous and hyperaemic synovial membranes with increased joint fluid and erosions of the joint cartilage.

Histologically there is proliferation of the synovial lining cells with synovial villous hypertrophy, and a diffuse cellular infiltrate consisting mainly of lymphocytes, but also macrophage/monocytes and plasma cells(Oliver et al. 1981; Cutlip et al. 1985; Narayan et al. 1992; Watt et al. 1992b).The synovial fluid contains a raised level of all lymphocyte subsets, although $CD8^+$ cells predominate and there is a reversal of the $CD4^+/CD8^+$ ratio (KennedyStoskopf, 1989; Harkiss et al. 1991). A similar pattern is evident in the synovium of the affected joints, with a predominant $CD8^+$ response (KennedyStoskopf, 1989; Anderson et al. 1994). An increased level of MHC class II expression has been found in both the synovial fluid and synovial membrane (KennedyStoskopf, 1989; Harkiss et al. 1991; Anderson et al. 1994).

The $CD4^+/CD8^+$ ratio was also considered to be reversed in the peripheral blood of infected animals in one small study(KennedyStoskopf, 1989), a finding that was challenged subsequently in a considerably larger study (Lujan et al. 1993) who considered there to be no significant differences in the levels of circulating lymphocytes using a range of monoclonal lymphocyte markers.

1.3.5 Diagnosis and control

The current methods of detection of *in vivo* infection with MVV rely on serological testing for the presence of antibodies to MVV proteins, with both the agar gel immunodiffusion test (AGIDT) and enzyme linked immunosorbent assay (ELISA) in common use (Winward et al. 1979; Houwers, Gielkens, 1979). One comparative study discovered little difference between the accuracy and sensitivity of these methods (Dawson, 1982), although more recently several authors have claimed a greater sensitivity for ELISA techniques, especially those relying upon whole virus

preparations (Houwers et al. 1982; Kwang, Torres, 1994; Rosati et al. 1994; Zanoni et al. 1994).All these methods are reliant on the presence of circulating antibodies, which has been shown to underestimate the number of virally infected individuals as determined by in-situ hybridisation (Johnson et al. 1992). Detection of individual infected cells is possible using the polymerase chain reaction in the fixed cell, followed by detection with in situ hybridisation (Haase et al. 1990). This markedly improves sensitivity, but would prove difficult to operate as a true diagnostic test as it is a costly and complicated technique.

A recent study has suggested the use of cerebrospinal fluid (CSF) for the diagnosis of visna, relying on cellular and globulin changes that may be specific to the disease (Lowenthal, Karcher, 1994).

Control of spread of the disease is complicated by the prolonged latent period of clinically inapparent infection, during which time virus shedding and transmission may occur, and the dependence on serological testing for identification of infected individuals. Vaccination against MVV has been studied in detail, but the persistence of infection as a DNA provirus, antigenic variation within the virus population, transmission to neonates, and a poorly understood immune response to live viral challenge all create practical difficulties that are unlikely to be overcome in the foreseeable future (reviewed by Pearson et al. 1989). Current methods of control rely on serological testing and the culling of either entire flocks or infected individuals and their offspring (De Boer et al. 1979; Houwers et al. 1987). The removal of lambs from infected dams at birth has also proved effective as a control measure, but is difficult to achieve in commercial flocks (De Boer et al. 1979). Within the U.K., a voluntary national Sheep and Goat Health Scheme is in operation which accredits flocks that have been confirmed free from infection serologically.

Currently there is no specific clinical treatment available.

1.3.6 Economic importance

The level of economic loss associated with MVV is difficult to assess due to the difficulty in diagnosis and the insidious onset of clinical signs which tend to lead to poor production, emaciation and premature culling. The occurrence of indurative mastitis has been linked with poor growth rates in lambs (Pekelder et al. 1994), and the economic loss associated with infection has been recently estimated at £5 per ewe in a flock (Dawson, 1994).

1.4 INFECTION AND THE IMMUNE SYSTEM

1.4.1 Response to viral challenge

Due to the uncertainty surrounding the epidemiology of natural infection, the response of the immune system to viral challenge has been largely studied using experimental infection models. These have consisted of viral innoculation via the respiratory route, directly into central nervous tissue, and via the subcutaneous route.

The immune response to the virus is composed of both cell mediated and humoral components. The immune response to an acute viral challenge administered into the respiratory system, mimicking the supposed method of natural disease transmission, has been evaluated (Sihvonen, 1981; Larsen et al. 1982b; Lairmore et al. 1986; Cordier et al. 1992; Begara, 1994). All these studies have reported an early increase in lymphocyte numbers in bronchoalveolar lavage fluid (BALF), with

phenotyping of lymphocyte subsets being performed in two studies (Cordier et al. 1992; Begara, 1994). These studies have provided conflicting results, with Cordier et al reporting a significant increase in both the $CD4^+$ and $CD8^+$ lymphocytes, significant increase in PMN numbers, and unchanged macrophage numbers in BALF at three months post infection, and Begara reporting a decrease in $CD4^+$ and increase in $CD8^+$ lymphocytes (with resultant inversion of the $CD4^+/CD8^+$ ratio), decreased PMN numbers, and decreased macrophage numbers in BALF at 4-8 weeks post innoculation. Seroconversion to MVV antigens has been found to occur in a majority of innoculated sheep at 2-12 weeks post challenge, and to persist throughout the study period (Larsen et al. 1982b; Lairmore et al. 1986; Begara, 1994). The induction of a viral-specific lymphocyte response, as assessed by the lymphocyte transformation test, was found to occur at 3 weeks post innoculation, but had returned to baseline by 7 weeks post innoculation (Larsen et al. 1982a).

Other workers, particularly those interested in the pathogenesis of the neurological form of the disease visna, have utilised direct viral innoculation intracerebrally (Petursson et al. 1976; Griffin et al. 1978; Oliver et al. 1981). All studies reported the finding of visna like pathological lesions in the brains of infected sheep, although there was disparity in the results of leukocyte counts in the cerebrospinal fluid (CSF); one study reports a slight increase in leukocyte count in a small number of innoculated sheep (Oliver et al. 1981), whilst the other two report an initial transitory marked elevation in leukocyte numbers in the CSF followed by a prolonged marginal elevation throughout the period of study (Petursson et al. 1976; Griffin et al. 1978). All three studies report the development of a serological antibody response, usually detected at 1-2 months post infection.

The intra-articular innoculation with virus is reported to produce a mononuclear cell infiltrate into the synovial fluid, with a lymphocytic infiltration in the synovial membrane (Oliver et al. 1981). A seroconversion was found to occur between 14 and 42 days post innoculation.

Finally, intradermal viral innoculation has been undertaken (Bird et al. 1993; Blacklaws et al. 1994; Blacklaws et al. 1995), allowing the investigators to study changes in cell populations in the regional lymph node and efferent lymphatics draining the site of the experimental infection. Viral challenge led to the initial decrease in cell numbers in efferent lymph, with a subsequent return to baseline cell output, but with an increase in $CD8^+$ MVV specific cytotoxic cells, the presence in draining lymph nodes and the peripheral circulation of MHC restricted cytotoxic cells, and circulating anti-viral antibody production (Bird et al. 1993; Blacklaws et al. 1994; Blacklaws et al. 1995). Virus was only rarely isolated from affected tissues.

1.4.2 Persistence of the virus

The persistence of the virus in the face of both an antibody and cell-mediated immune response is a phenomenon that is not fully understood, and accounts for the continually progressive nature of the clinical course of disease. The favoured explanation for this viral persistence is the restriction of viral gene expression by the host cell, with the virus existing in a proviral from in the monocyte and only becoming active when the cell is activated (Narayan et al. 1982; Gendelman et al. 1986). This leads to the situation where only a relatively small proportion of cells that contain viral protein express any viral proteins on their cell surface (Brodie et al.

1995), and hence the immune system is unable to recognise the vast majority of infected cells.

Another possible mechanism involves the mutation of the virus under pressure from circulating antibodies, leading to production of strains of virus that are no longer neutralised (Zink et al. 1987).

1.4.3 Immunosuppression

In contrast to the profound immunosuppressive activity of the other members of the *Lentivirinae* family, there has been little documented evidence of a similar alteration in the immune response of sheep infected with MVV. There have been suggestions that MVV infection is associated with increased infection with SPA (Myer et al. 1988) and lungworms (Giangaspero et al. 1993), but the direct association through immunosuppression is not clear and this may be a synergistic relationship (Dawson et al. 1985; Gonzalez et al. 1993). More direct evidence of immunosuppression was provided when it was discovered that there was a depression of the gross dimensions of the delayed type hypersensitivity response in sheep infected with MVV (Myer et al. 1988).

1.4.4 *In-vivo* testing of the cell mediated immune system

The cell mediated immune response in ruminants consists of complex interactions between antigen presenting cells and antigen specific T lymphocyte clones, particularly of the $CD4^+$ phenotype (McKeever, 1994), and is particularly

responsible for immune responses to certain agents such as viruses and fungi and mycobacteria (Kniker et al. 1984). Although there have been many recent developments of in vitro tests of the immune system, comparative studies have indicated that the most readily undertaken and reliable indicator of the ability of the cell mediated immune system to function *in vivo* is the delayed type hypersensitivity (DTH) response (Spitler, 1980; Ahmed, Blose, 1983; Borleffs et al. 1991; Blatt et al. 1993; Otto et al. 1993; Rosentreich, 1993). This test consists of the intradermal injection of a specific antigen into a previously sensitised individual.

The assessment of the cell mediated system is of particular importance in lentiviral infection, where there is a dysregulation of this part of the immune response, and the presence of virus in at least one of the component cell phenotypes. As such, the gross size of the DTH has been used extensively in HIV infection as a marker of the degree of immunosupression in individual patients, and is a component of the widely utilised Walter Reed system of assessment of progression to acquired immune deficiency syndrome (AIDS) (Redfield et al. 1986). A similar pattern has been described in cats infected with FIV (Otto et al. 1993), and sheep infected with MVV (Myer et al. 1988). There has been no histological or mechanistic assessment of this reported decrease in the gross size of the DTH.

1.4.5 Maedi visna as an animal model for HIV infection

The thorough investigation of pathogenic mechanisms is reliant on both the collection of biopsy material for further in vitro experiments, and direct invasive experimentation in the living animal. The study of human subjects is obviously highly restricted due to both ethical considerations and the danger of transmission of

infection to the experimenter. For these reasons, the study of animal models of human disease provides useful insights into pathogenesis, and helps direct the investigation of the human condition. The sheep is inexpensive to purchase and maintain, docile and readily handled, and presents little risk of zoonosis to the handler. For these reasons, the sheep has become an important experimental animal (Borrie, Mitchell, 1960).

Considering lentivirus infections in particular, the ovine lentivirus shares many features of the human equivalent: genomic organisation, method of viral replication, antigenic variation, restriction of replication the host cell, persistence of the virus in the face of humoral and cell mediated immune responses, slow and progressive development of disease, and similarities in the pathological lesions produced by the disease (Narayan et al. 1988; Petursson et al. 1989; Petursson et al. 1991). The major difference between the two viruses rests on the concurrent infection of $CD4^+$ lymphocytes, as well as macrophages, in HIV. This difference appears to be the important factor in producing the profound immunosuppression found in HIV, and is only apparent at late stages when the development of secondary infections tends to obscure primary pathology (Narayan et al. 1988; Petursson et al. 1989; Petursson et al. 1991). Study of the pathogenesis of Maedi-Visna, particularly in vivo, is therefore of comparative interest in the understanding of the human HIV infection.

1.5 THE DELAYED-TYPE HYPERSENSITIVITY (DTH) RESPONSE

The DTH response was first recognised by Koch, who discovered the now classical response to the intradermal injection of tubercle bacilli extract, in which human patients or guinea pig models infected with tuberculosis reacted with an inflammatory lesion characteristic of the tuberculous inflammatory process which

was maximal at 48-72 hours post injection. Further work established that this reaction could take place in the absence of antibodies, and that the sensitivity could be transferred by means of blood cells (reviewed by Spitler, 1980), leading to the description of this reaction as 'cell-mediated immunity'. The reaction has now been reproduced utilising a variety of antigenic materials (Rosentreich, 1993).

In most instances, the DTH is evaluated purely at a gross level, with the increase in skin thickness at the site of the indurative response being measured. In this form the reaction is utilised in a variety of clinical situations; either as an aid to the diagnosis of infectious disease such as tuberculosis, or as an assessment of the functional ability of the cell mediated immune system *in vivo* (Spitler, 1980; Ahmed, Blose, 1983; Otto et al. 1993; Rosentreich, 1993; Pesanti, 1994).

A large range of DTH sensitisation and elicitation models have been previously reported, leading to the possibility that there could be an equally wide range of mechanisms involved in producing such a lesion. Indeed, a recent study comparing the DTH responses to either tuberculin or human albumin in mice discovered that there are two distinct cellular mechanisms operating; the tuberculin reaction being macrophage dependent and the albumin reaction being mast cell dependent (Torii et al. 1993). Many previous studies have concentrated on the chemically induced skin contact hypersensitivity reaction (Nickoloff et al. 1990), and while this model was considered for use in the present studies, the marked immunological involvement of the epidermal cells in this response led to the consideration that it would be difficult to evaluate the results terms of specific macrophage cell functions that were the basis of this study into the effects of MVV infection on the DTH reaction. The tuberculin reaction is also a commonly investigated example of the DTH response and appears to operate in a macrophage dependent manner. After careful consideration, this

model was selected to be the experimental system of choice, and this review will concentrate on the tuberculin reaction.

In order for a hypersensitivity response to take place , the subject has to be first sensitised to a particular antigen (Imamura et al. 1993; Ng et al. 1995). In the case of the tuberculin response, sensitisation is commonly performed by vaccinating the subject with a *Mycobacterium* vaccinal preparation (Davey, 1995). The response may then be generated by the injection of bacterial proteins extracted from cultures of *Mycobacterium*, most commonly into the dermis (Davey, 1995). The most frequently used bacterial preparation is the purified protein derivative (PPD) of *Mycobacterium bovis*, which is often referred to as tuberculin (although the term tuberculin appears to be used somewhat indiscriminately). PPD is prepared from cultures of *Mycobacteria* by initially heat killing the culture and then harvesting the cell free supernatant. This supernatant is then precipitated with trichloroacetic acid (Klausen et al. 1994). As such, PPD is a crude mixture of a large range of antigenic material including heat shock proteins (Vordermeier et al. 1992). Attempts have been made to identify the components of PPD responsible for the DTH, and one study found a 38kDa protein capable of eliciting a DTH response in immunised mice (Vordermeier et al. 1992). There has been no such reported work undertaken in the sheep, and the author obtained a supply of a number of pure proteins which were components of PPD and had been shown to produce immunological responses in other animal species (Hewinson, personal communication). Unfortunately, these proteins failed to elicit any detectable DTH response when injected into sensitised sheep, and PPD as opposed to a single protein, remained the sole choice for DTH induction in this species.

The histological nature of the DTH reaction has been the subject of study for some number of years, with initial controversy regarding the cellular characteristics of the reaction. One of the earliest studies (Dienes, Mallory, 1932) concluded that the predominant infiltrative cell type was of mononuclear morphology, but was challenged by two reports (Feldman, Fitch, 1937; Long 1937) which reported the PMNs as the dominant cell type found in the early (up to 24 hour time point) reaction, subsequently being replaced by the mononuclear cells. The main site of accumulation of the PMN was found to be perivascularly and perineuronally. A marked accumulation of oedema fluid, which has subsequently been shown to be involved in the production of the induration in the lesion (Muller, 1995), was consistently reported. Subsequent to the studies by Feldman and Fitch (1937) and Long (1937), an attempt was made to recreate the initial work of Dienes (Follis, 1940). This provided a conflicting result to that initially reported by Dienes, and confirmed the work of Feldman and Long in concluding that the predominant cell type in the early (up to 24 hour) reaction was the PMN. The original work of Dienes was again repeated (Gell, Hinde, 1951) and this time the presence of a significant number of PMNs in the early reaction was recorded, although the authors considered these to be a secondary and non specific response to epidermal necrosis and persisted with the view that the mononuclear cells were of primary importance in the reaction. Similar observations were made by Boughton and Spector (1963), with PMNs considered the main cell type in the early reaction, located mainly in a perivascular pattern, being superseded latterly by mononuclear cells. Another interesting finding of this study was that non-sensitised animals differed from sensitised animals in their lack of marked PMN migration in the early reaction.

The importance of the mononulcear cells to the DTH reaction was subsequently demonstrated experimentally by the passive transfer of reactivity to

tuberculin, from a sensitised to a naive animal using these cells (Turk, Oort, 1963). The specificity of the cells present in the DTH lesion was further studied utilising radioactive labelling techniques, but it was found that the origin (donor or recipient) of the mononuclear cells migrating into the DTH lesion was not significantly different from that of cells migrating into non specific control lesions (Turk, Oort, 1963). The same study also noted the presence of large numbers of PMNs in the early lesion. The actively dividing nature of the invading mononuclear (described by the authors as lymphocytes morphological grounds) was then confirmed by a study of tritiated thymidine uptake by cells in a DTH lesion, with lymphocytes exhibiting a marked uptake of the radioactive material indicative of active cellular division (Kosunen at al. 1963). Several more recent studies have also indicated the presence of large numbers of PMNs (Legendre et al. 1979; Platt et al. 1983; Norris et al. 1991), although the most recent publication regarding PMN influx (Gao et al. 1994), suggested that PMNs did not participate in the lesion. This work unfortunately was confined to time points after 24 hours when most authors suggest PMN migration has ceased.

Attempts were then made to differentiate the mononuclear cell types involved in the lesion, initially using histochemical techniques. Differentiation of macrophages from lymphocytes was performed using an acid phosphatase staining method (Turk et al. 1966). This study indicated that macrophages and lymphocytes were present in roughly equal numbers up until 24 hours, at which point there was a marked lowering of macrophage numbers in the lesion with a corresponding increase in the levels of lymphocytes. PMNs were also found to be present in the early lesion in significant quantities. The advent of the use of antisera for the staining of histological sections facilitated the discovery of a marked deposition of extravascular fibrin in the reaction (Colvin et al. 1973). This was followed by the advent of the

monoclonal antibody, and its particular usage for the precise immunohistological staining of specific cells as defined by their expression of certain cell surface markers. Several workers reported the relative contribution of the T helper ($CD4^+$) and T suppressor ($CD8^+$) T lymphocyte subsets, with the $CD4^+$ being found to be predominant in the lesion, although the ratios of the cell types was found to be of the same order in the lesion as in the peripheral circulation which was interpreted as indicating a non selective or random migration of the T cell subsets into the lesion (Poulter et al. 1982; Konttinen al. 1983; Gibbs et al. 1984), with several of these authors indicating a large influx of macrophage marker expressing cells into the later lesion (Poulter et al. 1982; Konttinen et al. 1983; Platt et al. 1983).

More detailed work has followed in an attempt to unravel the mechanisms controlling the influx of cells into the lesion, with the discovery that the adhesion molecules endothelial adhesion molecule-1 (ELAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are associated with the influx of both PMNs and lymphocytes (Norris et al. 1991; Silber et al. 1994a; Silber et al. 1994b), and that the lymphocyte associated adhesion molecules very late antigen-4 (VLA-4) and lymphocyte function antigen-1 (LFA-1) are similarly involved in migration of mononuclear cells into the lesion (Issekutz, 1993).

The levels of cytokine expression in the DTH lesion have been evaluated indicating that in the early reaction interleukin-1 and TNF-α predominate, with interferon-γ and interleukin-2 expression (typical of the T-helper type 1 response) being also upregulated at the 24 hour time point (Chu et al. 1992; Tsicopoulos et al. 1992; Tsicopoulos et al. 1994).

In summary, there still remains controversy as to the cellular components of the DTH reaction, with PMNs being commonly found in the early reaction, but with many commentators considering the subsequent influx of mononuclear cells to be of more importance. The precise nature of the mononuclear cell influx is also a subject of controversy, most experimenters discovering the presence of considerable numbers of T lymphocytes, mainly of the $CD4^+$ subset, in the later lesion. There is discrepancy, however, in the reports of the numbers of macrophages in the later lesions, some commentators considering them the predominant cell in the later reaction, while others report a marked reduction in macrophage numbers at later time points. It should be pointed out that none of these studies were performed in sheep, and that there is no reference in published literature to the normal components of the DTH in sheep.

1.6 AIMS OF THE THESIS

This thesis sets out to confirm sheep chronically infected with MVV (as defined by persistent seropositivity) exhibit depressed DTH responses, and attempts to characterise the altered response shown by these animals. This has both relevance in furthering the study of the pathogenesis of a disease which is of economic importance to the sheep breeding industry, and has implications for the understanding of the immunopathogenesis of the depressed DTH that has been recorded, but never histologically evaluated, in the human disease AIDS. The primary step is the definition of the immunohistological characteristics of the normal DTH reaction in sheep, followed by the examination of the reaction in sheep infected with MVV and the definition of any abnormality discovered.

CHAPTER TWO

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

Texel sheep were purchased from a commercial flock with a history of clinical disease related to MVV and a high rate of MVV seroprevalence as previously described (Watt et al. 1992a). All animals were seropositive at the time of purchase, and were subsequently maintained in penned groups of approximately 30 sheep within a 'polytunnel' sheep house. The pen sizes were such that sheep were maintained in close contact with their peers at all times. They were managed in a conventional manner, with a feeding regime of hay supplemented with a commercial concentrate ration. Regular anthelmintic treatment was undertaken.

Sex and age matched control animals were purchased from pedigree Texel flocks that were participants in the MAFF sheep and goat health scheme, indicating that they had been serologically free from MVV infection for a considerable period of time. They were separately housed, but maintained in an identical manner to the infected sheep. These sheep were regularly serologically tested to confirm their seronegativity.

2.2 SEROLOGICAL TESTING

Sheep were regularly tested for the presence of serum antibodies to MVV antigen using an AGIDT supplied by The Central Veterinary Laboratory (Weybridge, UK) , which is the standard test used by MAFF in the UK (Winward et al. 1979). This test uses an antigen prepared from MVV ovine strain WLC-1 MVV. The kit

includes a positive control of ovine antiserum to an MVV 135 kDa glycosylated envelope protein, this being one of the main reactive viral antigens.

2.3 HAEMATOLOGY

Blood was collected by jugular venepuncture using a EDTA vacutainer tube system (Becton Dickinson and Co., Rutherford, USA) at the time of PPD challenge. The sample was haematologically analysed to provide total and differential white blood cell counts in a routine manner in an automated analyser by the Clinical Laboratory Service, Royal (Dick) School of Veterinary Studies, Easter Bush, Roslin.

2.4 INITIATION OF THE DTH

The experimental sheep were first injected intradermally in the flank region with five human doses (0.5ml) of a live attenuated Bacillus Calmette-Guérin vaccine strain of Mycobacterium bovis (Intradermal BCG vaccine BP, Evans Medical Limited) as routinely performed (J. Hopkins, personal communication). After an incubation period of 21 days, the DTH reaction was initiated by an intradermal injection of a PPD of Mycobacterium bovis (Central Veterinary Laboratories, Weybridge). A volume of 0.1 ml of PPD, equivalent to 0.01 mg of precipitable tubercular protein or 2500 international units of activity (assessed against an international in vivo assay utilising guinea pigs and cattle), was injected at selected sites in the medial thigh of each sheep. Standardisation was achieved via the use of a perspex template and spray marker to outline the injection areas; the precise point of injection being marked with a spot of permanent black ink. Contralateral control injections using an identical volume of sterile phosphate buffered saline (PBS) were performed in the opposite limb.

2.5 REACTION MEASUREMENT

The double skin thickness of the elevated lesion along with that of an adjacent unaffected portion of skin was measured using a set of measuring calipers (Camlab Ltd.). The increase in skin thickness of the lesion was calculated as the difference between the two values. The diameter of the lesion was measured using the calipers. On occasions when there was more than one lesion present at a particular time point, all lesions were measured and an average value for these two parameters recorded. Lesions were measured at the standard times of 2, 7, 24, 48, 72, and 96 hours post PPD injection.

2.6 SKIN BIOPSY TECHNIQUE

The skin area surrounding the lesion selected for biopsy was first aseptically prepared using Hibitane concentrate 5% (ICI plc, Cheshire, UK) at a dilution of 1 in 200 in 70% alcohol. The area around the lesion was then injected subcutaneously with the local anaesthetic agent Xylocaine 1% (Astra Pharmaceuticals, Kings Langley, UK) in order to produce a perilesional local anaesthetic 'ring-block'. An elliptical piece of skin including the DTH reaction was then removed with standard surgical instruments and the wound closed with stainless steel surgical staples (Ethicon Proximate PYW35, Ethicon Ltd.).

Biopsies obtained in this manner were processed according to the particular use to which they would be put.

Biopsies intended for standard histological investigations were placed in 4% buffered formaldehyde solution to fix for a minimum of 48 hours. They were then embedded into paraffin blocks in a Tissue Tek 3 embedding console, before being sectioned at a thickness of 4 microns and placed onto standard glass slides.

Biopsies intended for immunohistochemical staining were embedded in O.C.T and snap frozen in a slurry of dry ice and isopentane (Tissue Tek ltd., UK). They were maintained at -80°C in a freezer prior to being sectioned in a cryostat at -20°C at a thickness of 6 microns and placed on slides coated with 'Biobond' tissue adhesive (British Biocell International Ltd., UK).

Biopsies intended for RNA extraction were wrapped in aluminium foil and snap frozen in liquid nitrogen. They were maintained in a freezer at -80°C until used for extraction.

2.7 HISTOCHEMICAL STAINING

2.7.1 Haematoxylin and Eosin staining

Sections were initially dewaxed in a graded series of decreasing percentage alcohols. They were then immersed in haematoxylin for one minute, washed for one minute in running tap water, immersed in eosin for 30 seconds, washed in tap water for one minute, and then dehydrated through a graded series of increasing percentage alcohols before being finally cleared in xylene. The stained sections were mounted in DPX mountant (BDH Ltd., UK) and covered with glass coverslips. This staining was performed on a Shandon Linistainer automated staining device (Shandon Ltd., UK).

2.7.2 Martius scarlet blue stain

Sections were initially dewaxed in a graded series of decreasing percentage alcohols and rinsed for several minutes in tap water. Subsequently, they were

immersed in celestine blue for 10 minutes, immersed in haematoxylin for 10 minutes, rinsed briefly in tap water, immersed in Scott's tap water substitute for 3 minutes, rinsed in tap water for 5 minutes, rinsed briefly in 95% ethanol, immersed in 0.5% Martius yellow in 95% ethanol with 2% phosphotungstic acid for 2 minutes, rinsed briefly in tap water, immersed for 15 minutes in 1% brilliant crystal scarlet 6R in 2.5% acetic acid, rinsed briefly in tap water, immersed in 2% phosphotungstic acid for 2 minutes, rinsed briefly in tap water, immersed in 0.5% soluble blue in 1% acetic acid for 4 minutes, rinsed briefly in tap water, and finally dehydrated through a graded series of increasing percentage alcohols, cleared in xylene and mounted in DPX mountant with a glass coverslip placed on top.

2.7.3 Toluidine blue staining

Sections were initially dewaxed in a graded series of increasing percentage alcohols and rinsed in tap water. They were subsequently immersed overnight in a 0.5% toluidine blue in 0.5N HCl solution, rinsed briefly in distilled water, rinsed briefly in 0.125N HCl, immersed briefly in isopropanol, cleared in xylene and finally mounted in DPX mountant with a glass coverslip.

2.8 IMMUNOHISTOCHEMICAL STAINING

2.8.1 Monoclonal antibodies utilised

The monoclonal antibodies utilised and their specificities are incorporated in the following table.

Code name	Specificity	Dilution	Reference
SBU-T4	Ovine CD4	1:1000	(Maddox et al. 1985)
SBU-T8	Ovine CD8	1:1000	(Maddox et al 1985)
86D	Ovine γδ T cell receptor	1:1000	(Mackay et al 1989)
SW73.2	Ovine pan MHC class II	1:100	(Hopkins et al. 1986)
VPM63	Ovine Fcγ receptor 2 (macrophage)	1:100	Gupta (personal communication)
VPM65	Ovine CD14 (macrophage)	1:100	Gupta (personal communication)
OM1	Ovine CD11c (macrophage)	1:100	(Gupta et al. 1993)
DU2.87	Ovine B cells	1:100	(Gupta et al. 1994)

2.8.2 Stain development

A 'Vectastain Elite ABC Kit' (Vector Laboratories Inc., Burlinghame, USA) immunohistochemical stain development kit was used to demonstrate the monoclonal antibody staining of the tissue sections, and all the reagents (with the exceptions of the antibodies and phosphate buffered saline) used were supplied as part of the kit.

Firstly, the cryostat sections were air dried for 2 hours at ambient room temperature, immersed in acetone for 5 minutes at 4°C in order to fix the tissue, air dried at ambient room temperature for 5 minutes, and then washed three times for 5 minutes in 5 mls of pH7.3 PBS at room temperature. Subsequently, 100µl of 1.5% normal horse serum was placed on the section for 15 minutes at room temperature. A 100µl volume of the appropriate monoclonal antibody at the appropriate dilution was then added to the section, and the slides were incubated for 12 hours at 4°C. The slides were subsequently washed three times for 5 minutes in 5 mls of PBS at room temperature, before 100µl of biotinylated link antibody (of suitable species specificity for the primary monoclonal antibody) was added for 30 minutes at room temperature. This was followed by a further three washes for 5 minutes in 5 mls of PBS at room temperature, before the addition of 100µl of a 0.3% solution of hydrogen peroxide in methanol for 30 minutes at room temperature. Three further washes for 5 minutes with 5 mls of PBS at room temperature followed, with the subsequent addition of 100 µl of ABC (avidin-biotin complex) enzyme linked reagent for 30 minutes at room temperature. A final three washes for 5 minutes with 5 mls of PBS were then performed, with 100µl of the enzyme reagent 3-amino 9-ethylcarbazole (AEC) being added for 10 minutes at room temperature. The slides were then washed with copious volumes of tap water, counterstained with haematoxylin for 45 seconds, washed again in tap water, immersed in Scott's tap

water substitute for 1 minute and finally rinsed in tap water. The slides were mounted in Immumount reagent (Shandon Ltd.) with standard glass cover slips. Relevant control sections with either no antibody or irrelevant antibody added were simultaneously developed.

2.9 FLOURESCENCE ACTIVATED CELL SORTING (FACS) ANALYSIS OF CIRCULATING LYMPHOCYTES

2.9.1 Collection of peripheral blood

Peripheral blood was collected from the jugular vein using a 'vacutainer' system into a tube containing lithium heparin anticoagulant (Becton Dickinson and Co., Rutherford, USA).

2.9.2 Preparation and staining of cells

Ten mls of the heparinised blood were mixed with a red blood cell lysis buffer (Appendix 2.9.2) that had been prewarmed to 37 °C. This was incubated for 5 minutes at RT, and then centrifuged at 450x g for 5 minutes at RT. The lysed red blood cells in the supernatant were decanted, and the cell pellet resuspended in 50mls of an ice cold solution of a wash buffer solution containing PBS, bovine serum albumin, heparin and sodium azide (Appendix 2.9.2). The cell suspension was then recentrifuged at 450x g for 5 minutes to repellet the cells. This last step was then repeated, before the cells were finally resuspended in 10 mls of the buffer solution. The concentration of cells in the resultant suspension was measured in a haemacytometer, and adjusted to be in the range of $2\text{-}4x10^6$ ml^{-1}.

A 50µl aliquot of this cell suspension (equivalent to $1x10^5$ cells) was added to a microcentrifuge tube, and 25µl of the monoclonal antibody at a dilution of 1:250 in PBS was added. A single tube was left without any monoclonal stain as a negative control, and to provide samples for 'gating' the FACS machine. This mixture was incubated at 4°C for 15 minutes, and then centrifuged at 450x g for 5 minutes. The supernatant liquid was decanted, and the cells resuspended in 400µl of the wash buffer solution, before being recentrifuged at 450x g for 5 minutes. This latter washing stage was repeated, before 25µl of a 1:1500 dilution of FITC conjugated anti-mouse IgG, A and M (The Binding Site, Birmingham, UK), was added to the tube and the cells resuspended. This cell suspension was incubated for a further 15 minutes at 4°C, before undergoing a further two washes as before. The cells were finally resuspended in 400µl of wash buffer and analysed in the FACScan instrumentation.

2.9.3 FACS analysis

FACS analysis was performed using a Becton Dickinson FACScan apparatus, running Consort 30 software (Becton Dickinson Immunocytometry system, Oxford, UK). Initially, a control unstained sample of cells was run through the machine in order to visualise the forward and side scatter characteristics of the total cell population. From this total profile, the cell population corresponding to the lymphocyte population was selected , and a 'gate' manually set surrounding this population. The details of the gate setting were recorded, and used for all analyses. Once the gate was set, samples were run through the machine with an appropriate fluorescence detector in operation to detect the population of cells that exhibited

positive staining with the monoclonal antibody. Data was analysed using the Consort 30 software package.

2.10 LUNG PATHOLOGY

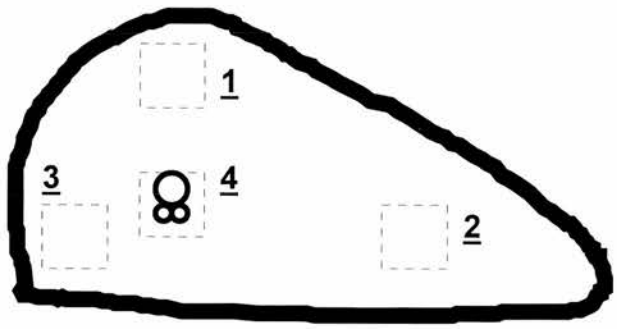
2.10.1 Post mortem technique

Animals which were used for postmortem studies were euthanased with an intravenous injection of pentobarbitone sodium BP (Lethobarb, Solvay-Duphar Veterinary, Southampton, UK) and immediately exsanguinated by sectioning the axillary blood vessels. A full routine post mortem examination was performed, and the lungs removed from the carcase, ensuring that the pleural membranes were left intact. The heart was dissected free from the pluck, and the isolated trachea and lungs were weighed. The left lung was then routinely fixed by the positive pressure airway instillation of phosphate buffered 4% paraformaldehyde, with a head pressure of 30 cm of paraformaldehyde applied until the tissue was completely fixed.

2.10.2 Sample selection

In order to provide standardised samples for the microscopic evaluation of the degree of lung pathology, a system of routine sampling was utilised. The diaphragmatic lobe of the fixed lung tissue was sectioned in the mid caudal area to produce an approximately 1cm thick slice. Four $1cm^2$ sections were then dissected from this slice in four anatomically distinct areas - 1. the dorsolateral parenchyma, 2. the ventral parenchyma, 3. the mediodorsal parenchyma, 4. the peribronchial region (Figure 1)

Figure 1. Indication of the standard sampling sites of the fixed lung



These samples were then embedded in wax, sectioned and stained with haematoxylin and eosin in an identical manner to that described for the skin biopsies (sections 2.6 and 2.7).

2.10.3 Quantification of lung pathology

The degree of pathological change present in the lungs of both MVV infected and control sheep was independently assessed subjectively by two experienced pathologists, with the slides being read in an observer blinded manner. The degree of interstitial reaction and smooth muscle hyperplasia were graded subjectively, being assigned a numerical value between 1 and 5, with the number of follicle like structures present in the section being counted. The degree of correlation between the observers was calculated.

2.11 ANTI-PPD ANTIBODY EVALUATION

2.11.1 Production of hyperimmune ovine anti-PPD serum

A control Texel sheep, that was normal on clinical examination, was used to produce hyperimmune anti-PPD serum. Firstly, a mixture of 1mg (equivalent to 1ml) of PPD and 1ml of complete Freund's adjuvant (Sigma-Aldrich Company Ltd., Dorset, England) were thoroughly mixed by repeated passage through a fine bore 23 gauge hypodermic needle attached to a syringe. The resultant homogenate was halved, and each half being injected into a separate site on the dorsal subcutis of the sheep. After a period of 4 weeks, a similar homogenate of 1ml of PPD in 1ml of incomplete Freund's adjuvant (Sigma-Aldrich Company Ltd., Dorset, England) was prepared and injected in two equal injections into distinct sites in the dorsal subcutis of the sheep. After a period of 2 weeks blood was collected from the sheep into sterile plain vacutainer tubes (Becton Dickinson and Co., Rutherford, USA). This blood was allowed to clot and then centrifuged at 2000rpm for 10 minutes to separate the serum, the resultant supernatant serum being harvested using a pipette and stored at -20°C prior to usage.

2.11.2 Negative control serum

As there are cross reactive epitopes between many Mycobacteria that sheep are normally exposed to in the environment and the BCG vaccine strain, negative control serum had to be obtained from an immunologically naive animal. Serum was therefore collected from blood samples that had been taken at parturition from hysterectomy derived lambs.

2.11.3 ELISA protocol

Firstly, 100µl per well of PPD antigen at a concentration of 10µg/ml of protein in PBS (equivalent to a dilution of 1 part PPD to 100 parts PBS/0.01% azide) was added to the wells of a 96 well, flat-bottomed, 'Immulon 1' ELISA plate (Dynatech Laboratories Inc., Chantilly, USA). The loaded plate was then wrapped tightly in aluminium foil and incubated for 12 hours at 4°C. The plate was subsequently washed vigorously three times in 4 litres of PBS containing 0.1% of tween 20. The test serum was then sequentially diluted with PBS containing 2% crystalline grade bovine serum albumin (BSA) (Sigma-Aldrich Company Ltd., Dorset, England) to produce a serum dilution series of 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, triplicate 75µl aliquots of each dilution added to the wells of the plate, with positive and negative controls. The plate was subsequently wrapped tightly in foil and left at room temperature for 30 minutes. This was followed by a further three vigorous washes in the PBS/0.1% tween mixture. Next, 100µl of alkaline phosphatase labelled anti-sheep IgG conjugate (Sigma-Aldrich Company Ltd., Dorset, England) diluted at 1:1000 in PBS/BSA2% was added to the test wells and incubated for 30 minutes at room temperature. The plate was then washed vigorously thrice in the PBS/0.1% tween solution. The enzyme substrate for the conjugated alkaline phosphatase was then freshly prepared immediately before use (Appendix 2.11.3). A 100µl quantity of this mixture was added to the test wells, and the plate was incubated at 37°c for 30 minutes and subsequently at room temperature until a noticeable colour change was apparent. The colour change was then quantified using an ELISA plate reader with a 405 nm filter in place.

2.11.4 Western blotting protocol

The initial stage of the western blotting technique was the separation of the PPD antigen into its protein constituents using a gradient SDS polyacrylamide gel electrophoresis (SDS-PAGE). This was performed using a Mini protean II electrophoresis apparatus in the following manner.

Firstly, all the glass plates and spacers were wiped with ethanol to remove all traces of grease and dirt. The plates, with interposed spacers, were located into the casting clamp and the gel solutions were freshly prepared (Appendix 2.11.4). A gradient gel pourer was then used to produce a continuous gradient gel, with the concentration of the gel passing from 5 to 20 %. Water was then gently overlaid over the gels using a pipette to fill the plates. The gels were left to set, at which point the overlay water was removed by inversion and blotting with filter paper. The freshly prepared stacking gel was then layered over the gradient gels with a pipette and an appropriate gel comb placed in position and left until the gel was set.

The PPD and a protein molecular weight marker were then prepared for running in this gel by mixing 1:1 with SDS dissolving buffer (Appendix 2.11.4). The resultant mix was then placed in a boiling water bath for 3 minutes.

The set gels were placed in a tris/EDTA/glycine/SDS running buffer (Appendix 2.11.4). The samples were loaded onto the gel using a Hamilton syringe, and the gel was run at 200V / 90mA until the dye had passed down the entire length of the gel

The gel was then removed from the apparatus in order that the proteins could be blotted onto a 'Hybond C' nitrocellulose membrane

First, the Tris/glycine/methanol blotting buffer was freshly prepared (Appendix 2.11.4), Four sheets of 'Whatman no.3' filter paper were soaked in this buffer and layered on the lower plate of a semi-dry electrophoresis apparatus (Anchos Ltd., UK), with a buffer soaked piece of nitrocellulose membrane placed on top. The stacking component of the gel was removed using a scalpel blade, and the gel was placed on top of the membrane, with the lane in which the marker was run clearly marked. A further four pieces of soaked 'Whatman no. 3' filter paper were placed on top of this gel. The upper plate of the apparatus was fitted, and an electric current of 120mA / 30V was applied for 2 hours. The nitrocellulose membrane was then removed from the apparatus. The marker lane cut off the membrane and stained with amido black stain for several minutes until clear bands were obvious, and subsequently destained in 50% methanol / 5% acetic acid to clear the background stain.

The remaining section of the membrane was placed in a PBS / 3% BSA solution, and agitated for one hour on an orbital shaker in order to block all protein binding sites. The membrane was then sectioned into strips, suitably sized to fit into a multichannel dish. The strips were appropriately marked, and then placed in a channel of the dish which contained the corresponding sheep serum which had been diluted 1:200 in PBS / 3% BSA. The dish was sealed with cling film and agitated for 12 hours at room temperature on an orbital shaker. The strips were subsequently washed thrice in situ with a PBS / 0.5% tween solution and then placed together into a staining jar.

A 1:1000 dilution of alkaline phosphatase conjugated anti-sheepIgG antisera (Sigma-Aldrich Company Ltd., Dorset, England) added, and were placed on an orbital shaker for 1 hour at room temperature. The strips were then washed three

times in PBS / 0.5% tween for 10 minutes on an orbital shaker at room temperature, and then immersed in 1M Tris pH9.5 for 10 minutes on an orbital shaker at room temperature.

The antibody binding was visualised by adding an alkaline phosphatase substrate ('sigma fast' tablets, Sigma-Aldrich Company Ltd., Dorset, England) to the strips until the colour reaction had developed At this point the reaction was stopped by flushing with copious amounts of tap water. The strips were dried with tissue paper and preserved in a darkened environment.

2.12 TISSUE RNA EXTRACTION

2.12.1 General handling procedures

The isolation of RNA suitable for reverse transcription and subsequent polymerase chain reaction (RT-PCR) is complicated by the fact that RNA is a relatively unstable molecule which is irreversibly degraded by the enzyme RNAase that is ubiquitously present on human skin, and by the fact that PCR is an extremely sensitive technique which detects such small quantities of nucleic acids that contamination and false positive results are major risks.

To combat these potential problems, all manipulations involving RNA were performed in a laminar flow hood, whilst wearing rubber gloves that were frequently changed, using new filtered pipette tips (Biorad Laboratories, Hercules, USA) for each sample, with disposable plastic labware or thoroughly cleaned and sterilised equipment, and with reagents specially prepared to be free from RNA and DNA degrading enzymes. To further prevent cross contamination, the isolation of RNA

and conversion of this into copy DNA (cDNA) were performed in a geographically distant laboratory from that in which the PCR of the copy DNA was performed, with no PCR product material being transferred back into the isolation and transcription laboratory.

2.12.2 RNEasy extraction

RNA was extracted from the tissue samples using an 'RNEasy' commercial RNA isolation kit (Qiagen Inc., Chatsworth, USA) according to the protocol supplied with the kit. Firstly, 10µl of β-mercaptoethanol was added per ml of the lysis buffer to prepare the buffer for use. A 600µl aliquot of this buffer mixture was placed in a sterilised tissue homogeniser which had been maintained at 0°C in ice, the tissue sample was removed from -70°C storage, and the tissue was immediately placed into the lysis buffer and homogenisation undertaken until the sample had been completely macerated. The resultant lysate was decanted into a microcentrifuge tube and centrifuged at 8000 x g for three minutes to remove tissue fragments. The supernatant was collected and mixed with an equal volume of 70% ethanol. This mixture was pipetted onto the RNEasy spin column, which was subsequently centrifuged for 15 seconds at 8000 x g. The flow through was discarded, 700µl of the wash buffer was pipetted onto the column, and the column was recentrifuged at 8000 x g for 15 seconds. A new 2 ml collection was fitted to the column, before 500µl of a working solution of 1 part wash buffer to 4 parts 100% ethanol was pipetted onto the column, which was recentrifuged at 8000 x g for 15 second. This wash was repeated, with the centrifugation prolonged to 2 minutes in order to dry the column. A new collection tube was then fitted and 15µl of RNA-ase free, sterile, distilled, tissue culture water (Sigma-Aldrich Company Ltd., Dorset, England) was pipetted onto the

column, before centrifugation at 8000 x g for 60 seconds. The flow through solution containing the RNA extraction was stored at -80°C until used for reverse transcription.

2.13 RT-PCR PROTOCOL

2.13.1 Reverse transcription

A reverse transcription mix was freshly prepared, taking care to maintain the temperature of the RT superscript below 0°C (Appendix 2.13.1).The isolated RNA was added to this mix in an eppendorf tube, mixed thoroughly and maintained at room temperature for 10 minutes. The tubes were then incubated for 60 minutes in a water bath maintained at 42'C. The RT reaction was then terminated by incubating the tubes for 5 minutes in a dry heating block maintained at 95°C. The tubes were then immediately plunged into ice and the cDNA stored at 4°C until required for the PCR reaction.

2.13.2 PCR protocol

The PCR reaction mix was prepared (Appendix 2.13.2). A 36.5µl volume of this mixture was then aliquoted into 0.5ml tubes and a drop of mineral oil (Sigma-Aldrich Company Ltd., Dorset, England) added as an overlay. A 3µl volume of the cDNA was subsequently pipetted into the reaction mix beneath the oil layer.

These reaction tubes were then transferred to a separate laboratory in order to minimise future contamination risks. The tubes were placed in a dry heating block

(Techne PHC 1) where they were heated at 95°C for 5 minutes, after which the temperature was rapidly reduced and maintained at 80°C. A 0.3µl volume of TAQ polymerase (Gibco BRL, UK) was then added to each sample beneath the oil layer.

The samples were then thermally cycled in a PCR thermal cycler (Techne PHC1) on a programme that had been temperature optimised for the particular cDNA molecule and primers being amplified. The cycle consisted of 35 cycles of a cycle consisting of : 95°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes, with a final extension phase of 72°C for 5 minutes.

2.13.3 Gel electrophoresis of PCR products

The PCR products were separated by an ethidium bromide/agarose gel electrophoresis method.

The gel was run in a TAE buffer, which was mixed as a 50 x concentrate (Appendix 2.12.3). Ten mls of this stock solution was diluted in 500 mls of distilled water immediately prior to use.

A 50 ml aliquot of this buffer was placed in a vessel with 1g of agarose and heated in a microwave oven to produce a clear solution. This was allowed to cool to approximately 50°C, and then 2.5µl of ethidium bromide was mixed into the solution before pouring into a adhesive tape sealed, ethanol cleaned gel trough, which was then left until the gel had cooled and hardened. Meanwhile, 22.5 microlitres of ethidium bromide were added to the remainder of the diluted running buffer.

Seven µl of the PCR product were then mixed with a loading buffer, taking care to that mineral oil was not included in the sample. A DNA ladder marker was simultaneously mixed with the loading buffer.

The samples were then added to the wells in the submerged gel and the gel run at 50mA for about 30 mins, at which point the coloured sample front had migrated approximately half way along the gel. The gel was then viewed with ultraviolet illumination and photographed, before undergoing southern blotting.

2.14 SOUTHERN BLOTTING AND DNA PROBING

2.14.1 Southern blotting protocol

The run gel was first washed in a 0.4M NaOH solution for 30 minutes on an orbital shaker. A gel sized piece of 'gene screen plus' nylon membrane (DuPont, Massachusetts, USA) was placed in distilled water, to which was added a small quantity of the NaOH solution, and the membrane was left to soak for 15 minutes at room temperature.

The blotting was performed in the minigel apparatus that had been used to run the gel, and 'Whatman no.4' filter paper was cut to provide one piece that had ends dipping into the gel tank troughs and 6 pieces that were the size of the gel. The larger piece and one or the other pieces of filter paper were soaked in 0.4M NaOH and placed in the gel tank. The washed gel was placed upside down on this filter paper, and the membrane placed on the upturned gel. Three further pieces of 0.4M NaOH soaked filter paper and two pieces of dry filter paper were added, followed by a large quantity of folded tissue paper. To complete the system, a flat glass plate was placed

on top of the entire apparatus and a large weight positioned on top of this plate. The gel tank troughs were then filled with 0.4M NaOH the apparatus was left for 12 hours at room temperature.

The membrane was then removed from the apparatus, and washed twice in an 2xSSPE buffer diluted 1:10 from a stock solution (Appendix2.14.1): The membrane was then placed on filter paper and crosslinked using an automatic programme in an ultraviolet crosslinking machine. The membrane was subsequently dried at room temperature, and stored in the dark in folded filter paper.

2.14.2 Radiolabelled oligonucleotide probing

The oligonucleotide specific for the internal sequence of the PCR product being probed was firstly radiolabelled with ^{32}P isotope using a polynucleotide kinase (PNK) enzyme to transfer this molecule from an ATP molecule to the oligonucleotide (Appendix 2.14.2). The unbound radioactive ATP molecules were then removed from the reaction mix using a sephadex column (Nick column, Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was first primed with TRIS-EDTA (TE) buffer at pH 7.4 (Appendix 2.14.2), before the PNK reaction mix was added and the radiolabelled oligonucleotide eluted by the addition of a further 400µl aliquot of TE buffer. The purified probe was then used in the hybridisation mix.

The hybridisation buffer solution required for probing were freshly prepared (Appendix 2.14.2).

The southern blotted membrane was placed in the chamber of a hybridisation machine (Techne hybridiser HB-1) with 50mls of 2xSSPE and revolved to remove bubbles. This solution was decanted and 20mls of hybridisation buffer was added and rotated for 30 minutes at 65°C. This solution was then decanted and a further 10mls of hybridisation solution was added to the chamber before 100µl of the radiolabelled probe was added to this solution. The chamber was then left to rotate for 12 hours at 65°C. The membrane was then washed twice with 20 mls of 2xSSPE for 5 minutes at room temperature, before being washed twice with 30 mls of 2xSSPE+1%SDS for 20 minutes at 60°C. Finally the membrane was washed in 0.1xSSPE at room temperature for 20 minutes before being wrapped in PVC film and left next to an X ray film in an X ray cassette for a variable period dependent upon the level of radioactivity present on the membrane as detected by a Geiger-Muller counter (usually a period of 12 hours was sufficient). The film was developed using an automated processor.

CHAPTER THREE

CHARACTERISATION OF THE NORMAL OVINE DTH REACTION

3.1 INTRODUCTION

There are relatively few previously published studies of the immunohistological characteristics of the PPD driven DTH reaction in any species, and no previously published work of this type in the sheep, although the reaction has been generated in sheep experimentally (Myer et al. 1988; Emery, Davey, 1995).

The purpose of this initial experiment, therefore, was to characterise the normal immunohistological characteristics of a classical tuberculin induced DTH using an ovine model. These data will provide the normal baseline for the studies into abnormal DTH responses that have been reported to occur in sheep infected with MVV (Myer et al. 1988).

3.2 MATERIALS AND METHODS

3.2.1 Animals

Six adult female sheep were used in this preliminary study (table 1). They were obtained from commercial sources, and were clinically normal. They were tested as serologically free from MVV infection using the AGIDT described previously (2.2).

Table 1

Sheep Number	Breed	Age (years)	Sex
111	Finn x	2	Female
114	Finn x	2	Female
142	Greyface	Aged (>5)	Female
143	Greyface	Aged (>5)	Female
144	Greyface	Aged (>5)	Female
145	Greyface	Aged (>5)	Female

3.2.2 DTH initiation, measurement, and biopsy

The DTH was initiated, measured, and biopsied in the standard manner described in 2.4 - 2.6.

3.2.3 Immunohistopathological staining

Routine paraffin embedded sections were processed and stained with haematoxylin and eosin and toluidine blue as described in chapter 2.

Frozen sections were processed as described in chapter 2 and stained with monoclonal antibodies SBU-T4 (CD4), SBU-T8 (CD8), 86D (γδ T cell), DU2.87 (B cell) and OM-1 (CD11c, macrophage) (chapter 2).

3.2.4 Quantification of the sections

Initially, a histopathological assessment of the H&E sections was undertaken. This indicated that the infiltrating cells were concentrated in the periadnexal areas, with a diffuse infiltration of the superficial dermis. In order to successfully quantify this infiltration, a wholly random pattern of counting was not suitable, as there were distinct areas of concentrated cellular accumulation. It was therefore decided to count the two distinct areas of accumulation separately for each section, in a modification of a previously described method (Bos et al. 1987). This was achieved using a 10x10 square graticule (Graticules Ltd., Tonbridge, Kent) fitted to the eyepiece of a microscope, with the sections appraised at x200 magnification. The first stage in this process was the selection of an adnexal structure, which was then centred in the field of view of the microscope. Ten randomly chosen squares located at the periphery of the adnexal structure were then selected and the total number of cells of interest within or with borders touching the lower and right hand grid line of these ten squares was then counted. This count was repeated four times, which commonly constituted the maximum number of adnexal structures present on each section. The second stage of this counting was the counting of cells in the superficial dermis. In order to achieve

this, an area of superficial dermis was selected and the total number of cells of interest within or with borders touching the lower and right hand grid line of ten randomly chosen squares was then counted. This count was repeated four times (Figures 3.1 and 3.2).

Figure 3.1 The periadnexal count was performed by moving the slide to centralise the grid over an adnexal structure, Ten of the squares which were judged to be adjacent to the adnexal structure (as indicated) were selected using a random number table, and the total number of cells of interest within these squares were counted.

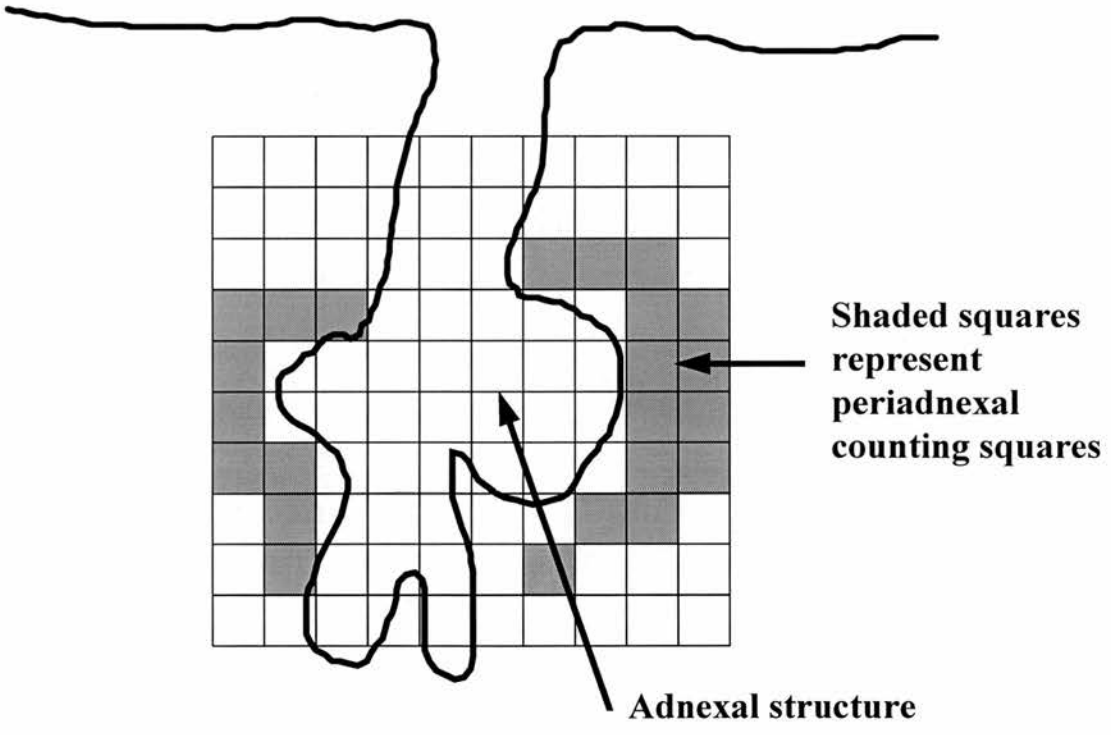
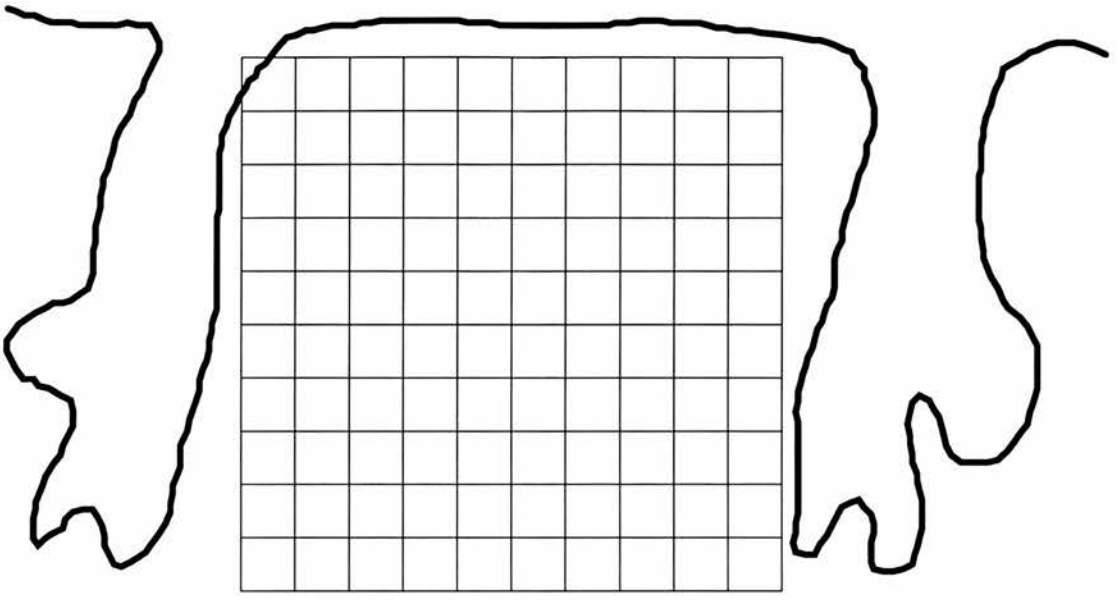


Figure 3.2 The superficial dermal cell count was performed by placing the grid over this area and selecting 10 squares using a random number table, The number of cells in these squares was counted, and the process repeated for a total of four counts.



Counting grid overlying superficial dermal area

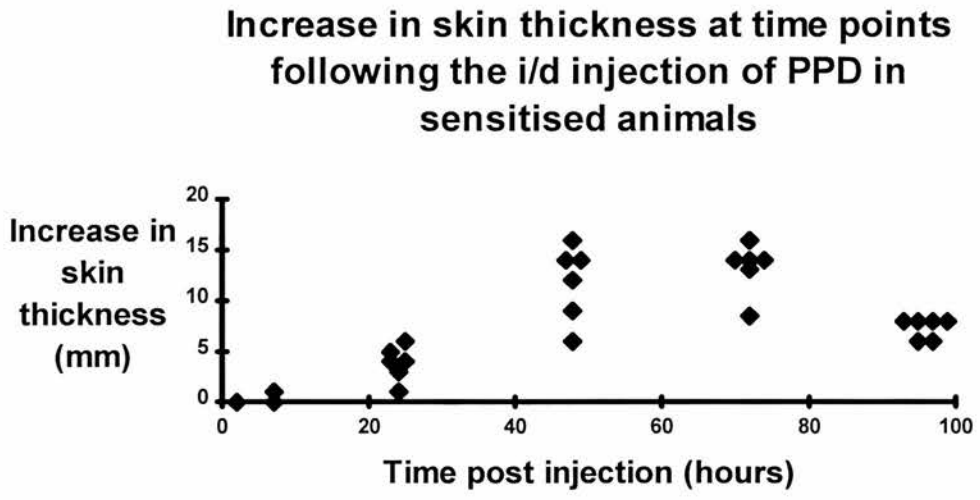
A consistent finding for all types of cells counted in this manner was a significant correlation (using a Spearman ranking statistic) between the superficial dermal and periadnexal cell counts.

3.3 RESULTS

3.3.1 Skin thickness

There was a marked reddened indurative swelling associated with the PPD injection site. This was maximal at 48-72 hours post injection, and was remarkably consistent between the sheep (Figure 3.3 and Appendix 3.3.1).

Figure 3.3



3.3.2 Histopathological assessment

Considering firstly the biopsies taken of the PPD antigen injected sites, the early 2 hour biopsy exhibited a mild dermal oedema with few PMNs marginating in the superficial dermal blood vessels of some animals (Figure 3.4). By seven hours, the marginated PMNs were present in all the biopsies examined, and there was migration of some of these PMNs into the perivascular spaces. Superficial dermal oedema was less marked (Figure 3.5). At the 24 hour time point, the reaction had become much

more florid, with a marked increase in cell density. The cells were primarily PMNs, but there was a significant increase in the number of lymphohistiocytic and fibroblast type cells. The reaction was mainly centred on the periadnexal structures, with a more diffuse superficial dermal infiltration. Superficial dermal oedema was evident, and fibrin was present in between the dermal collagen bundles (Figure 3.6). At the 48 hour time point, the number of PMN cells was markedly reduced, with an apparent increase in the number of cells of lymphoid morphology. Fibrin was present in the superficial dermis at high levels, and tissue oedema was marked, with dermal lymphatics becoming dilated with proteinaceous fluid. In several biopsies at this time point, there were large numbers of PMNs packed into the luminae of hypertrophic sweat glands. The cellular reaction remained centred on the periadnexal areas. The overlying epidermis frequently exhibited mild inter and intracellular oedema, although there were very few inflammatory cells present in this compartment (Figure 3.7). The reaction remained largely unaltered at the 72 hour time point (Figures 3.8 & 3.9). By the 96 hour biopsy, the reaction was becoming less cellular, with a decrease in oedema and fibrin levels. The cellular components remained at the same relative levels, with a mainly lymphoid cell component (Figure 3.10).

Biopsies taken from the control (PBS injected) sites, exhibited an early mild dermal oedema. There was no apparent cellular infiltrate present in any of the time course biopsies.

Figure 3.4: Photomicrograph of a 2 hour biopsy of the PPD lesion.. There are few cells in the dermis, with no evidence of infiltration. There is slight separation of the dermal fibres indicating a mild oedema associated with the injection of PPD (H&E, x100).

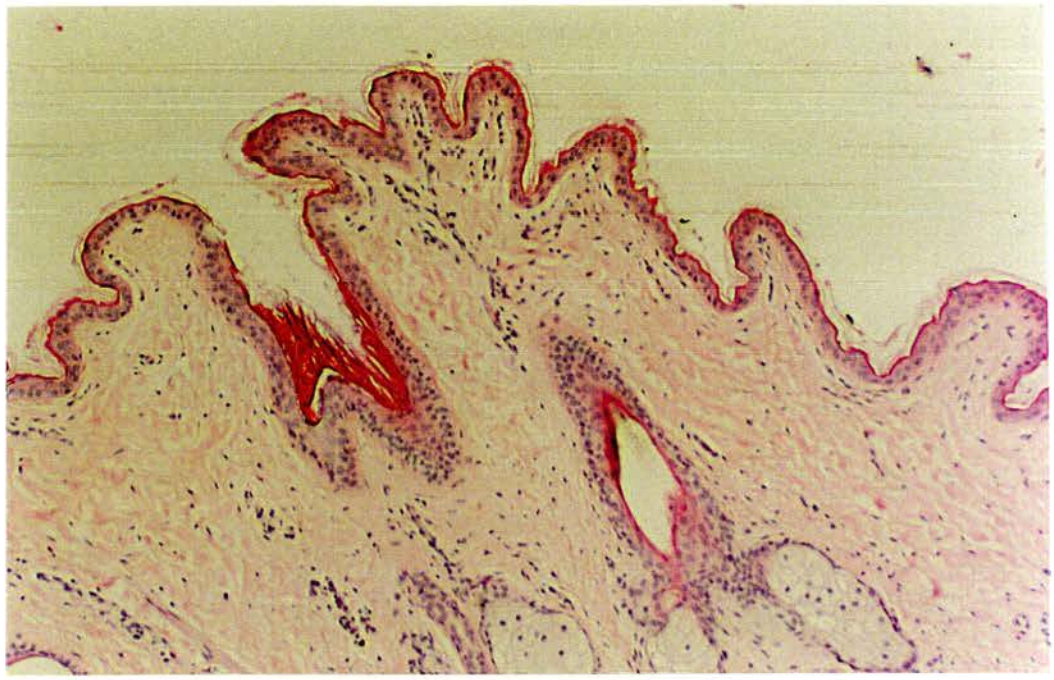


Figure 3.5: Photomicrograph of a 7 hour biopsy of the PPD lesion. There is still no widespread infiltration of the dermis, although blood vessels are more prominent and contain large numbers of marginating PMNs that are clearly visible on the inset photograph (H&E, x100 with inset x400).

Figure 3.6: Photomicrograph of a 24 hour biopsy of the PPD lesion. A marked dermal cell infiltration is apparent, especially concentrated around the adnexal structures. Most of these cells are PMNs. Dermal oedema and swollen lymphatics are also visible. (H&E, x100).

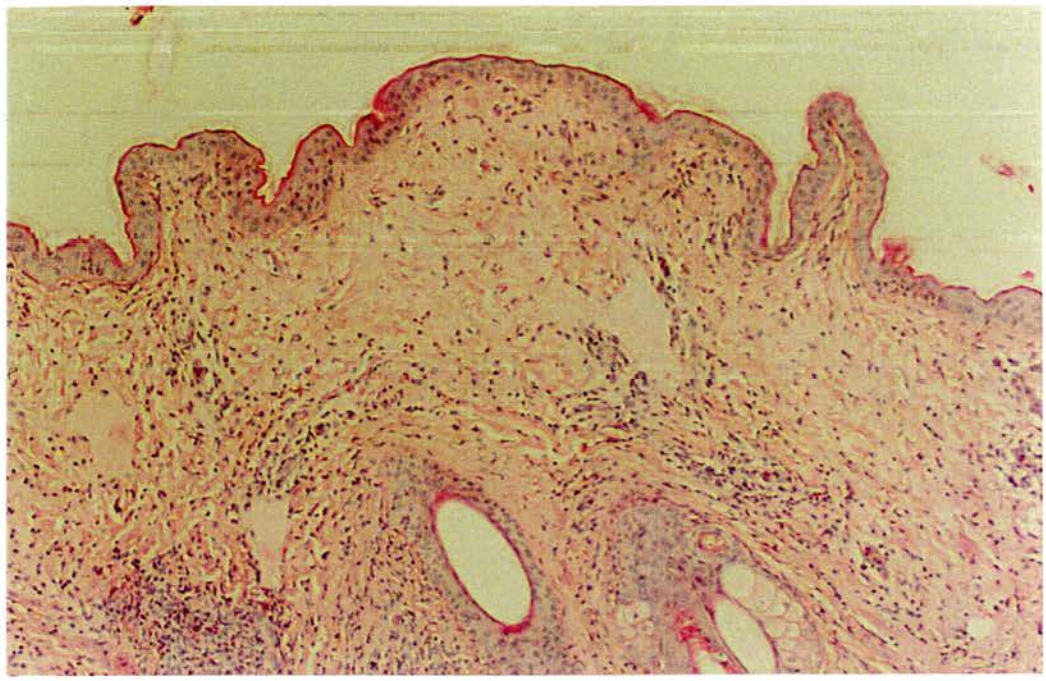


Figure 3.7: Photomicrograph of a 48 hour biopsy of the PPD lesion. There is a more marked dermal infiltration. These cells are of lymphoid morphology. Dermal oedema, proteinaceous fluid accumulation and swollen lymphatics are present. (H&E, x100).

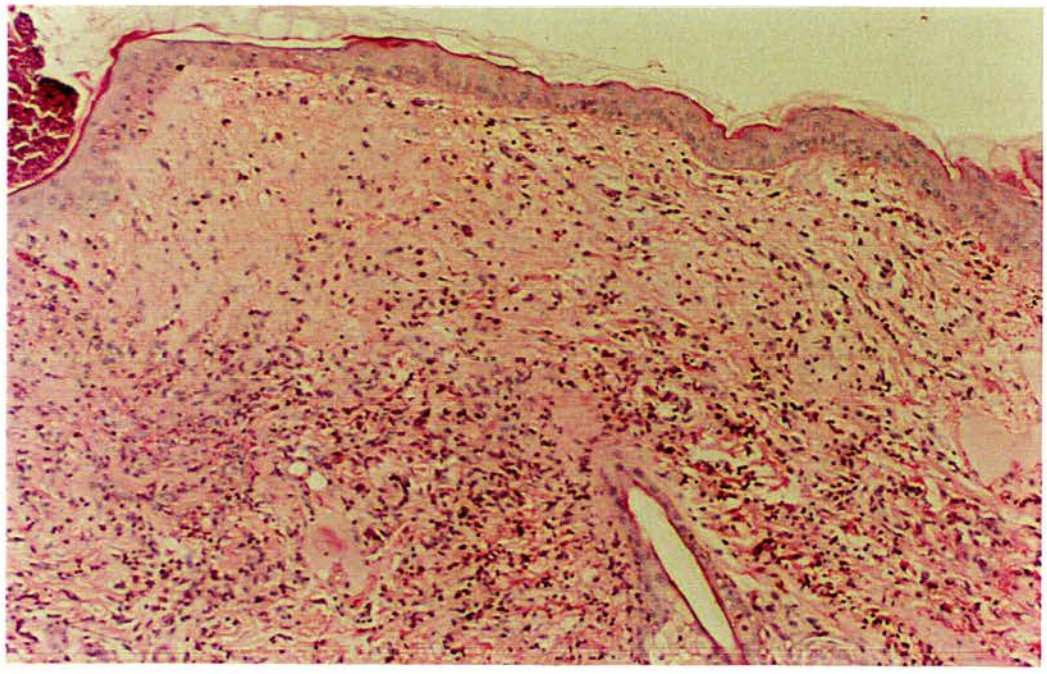


Figure 3.8: Photomicrograph of a 72 hour biopsy of the PPD lesion. At low power the concentration of infiltrating cells in a periadnexal position is clearly apparent. (H&E, x40).

Figure 3.9: Photomicrograph of a 72 hour biopsy of the PPD lesion. At higher power, cells are seen at a similar density to that in the 48 hour biopsy. A majority of these cells are of lymphoid type. Swollen lymphatics and dermal oedema are also apparent. (H&E, x100).

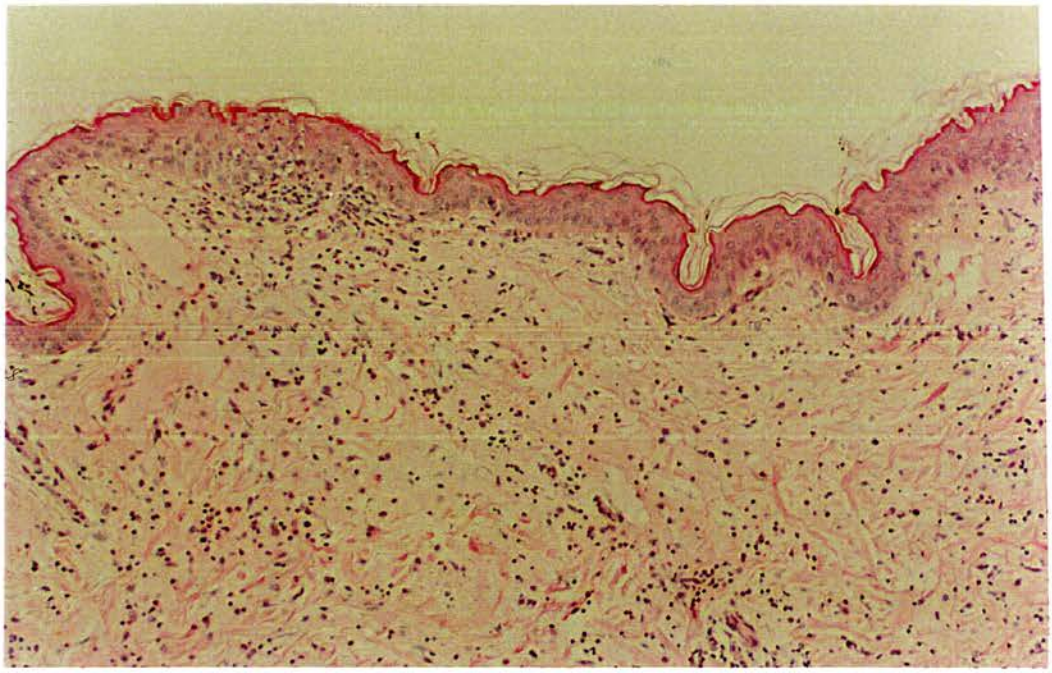
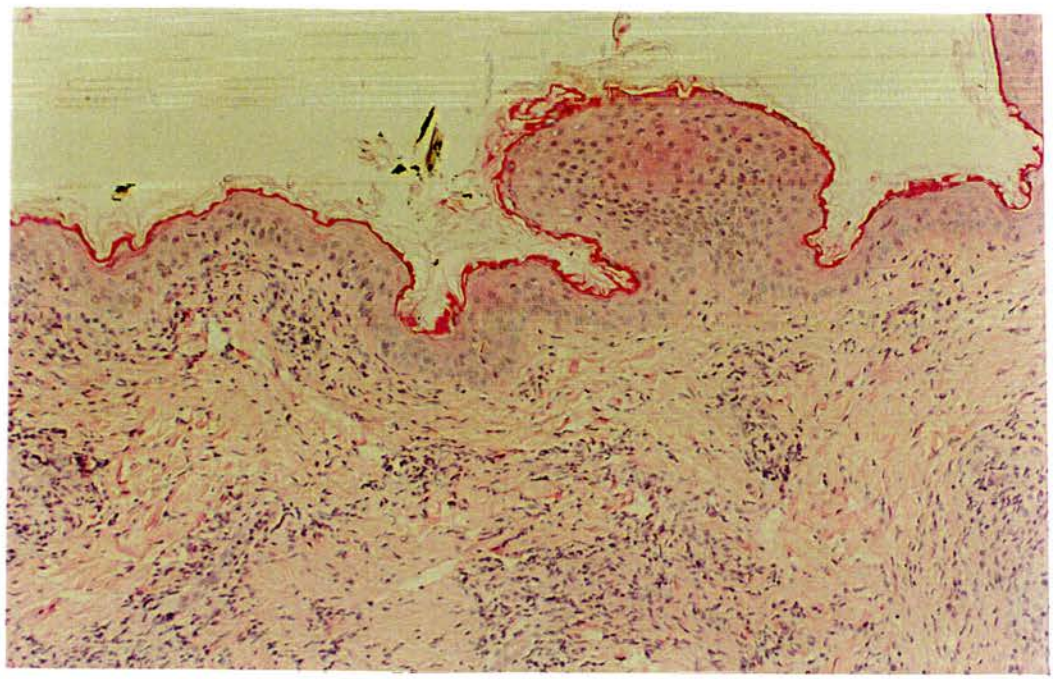


Figure 3.10: Photomicrograph of a 96 hour biopsy of the PPD lesion. Cell numbers and dermal oedema are beginning to subside by this time point. A majority of these cells are of lymphoid morphology (H&E, x100).



3.3.3 Polymorphonuclear neutrophil cell counts

In the antigen injected sites, the number of PMNs increased markedly in the early part of the reaction, reaching a maximal level at 24 hours post injection. This peak was followed by a dramatic decline in numbers to a figure of approximately half of this peak level by 48 hours post challenge. The numbers remained almost constant at the 72 hour time point, before declining to almost baseline levels by 96 hours post injection (Figures 3.17 and 3.18, Appendix 3.3.3).

The number of PMN cells in the control (PBS injected) remained unchanged throughout the time course of the study period (Figures 3.17 and 3.18, Appendix 3.3.3). Occasionally, small congregations of PMNs were seen surrounding a linear

track of tissue disruption. This was considered to be a non specific reaction to a 'needle-track' injury.

3.3.4 $CD4^+$ cell counts

In the antigen injected sites, the number of $CD4^+$ cells increased marginally at 24 hours post injection, before rising to an almost maximal level at 48 hours post challenge. This level increased slightly to become maximal at the 72 hour time point, before declining marginally at 96 hours post challenge. The maximal number of $CD4^+$ cells in the lesion was approximately half that of the maximal number of PMN cells (Figures 3.11, 3.12, 3.19, and 3.20, Appendix 3.3.4).

There were no significant changes in the $CD4^+$ cell counts in the control (PBS injected) biopsies (Figures 3.19 and 3.20, Appendix 3.3.4).

Figure 3.11: Photomicrograph of a 24 hour biopsy of the PPD lesion stained with a monoclonal antibody to $CD4^+$ (SBU-T4). A moderate number of positively cells are present, concentrated in the periadnexal area (AEC, x250).

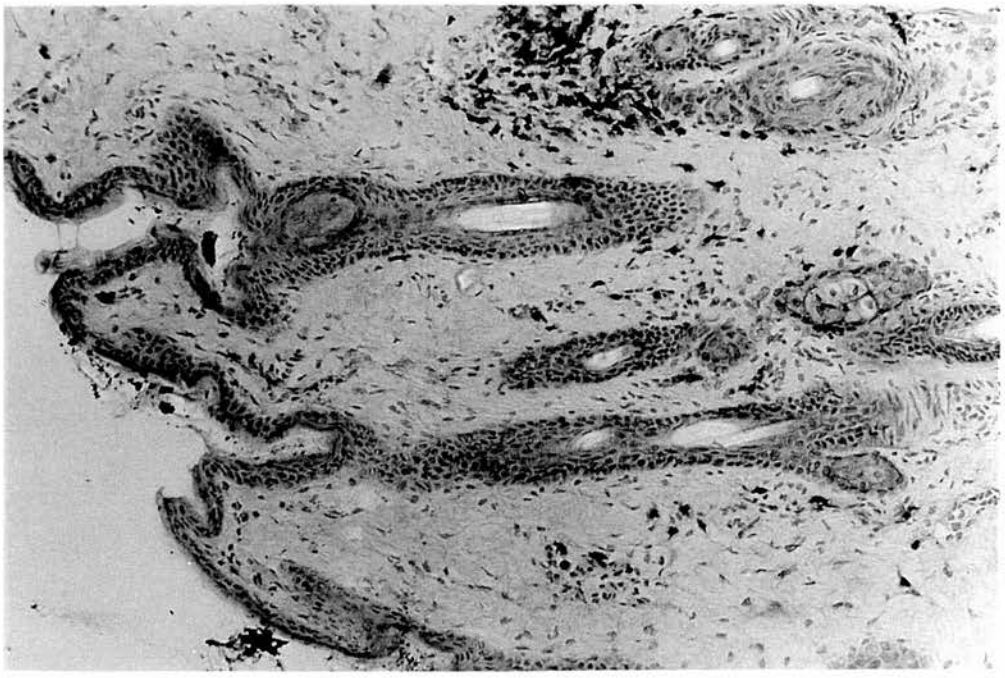
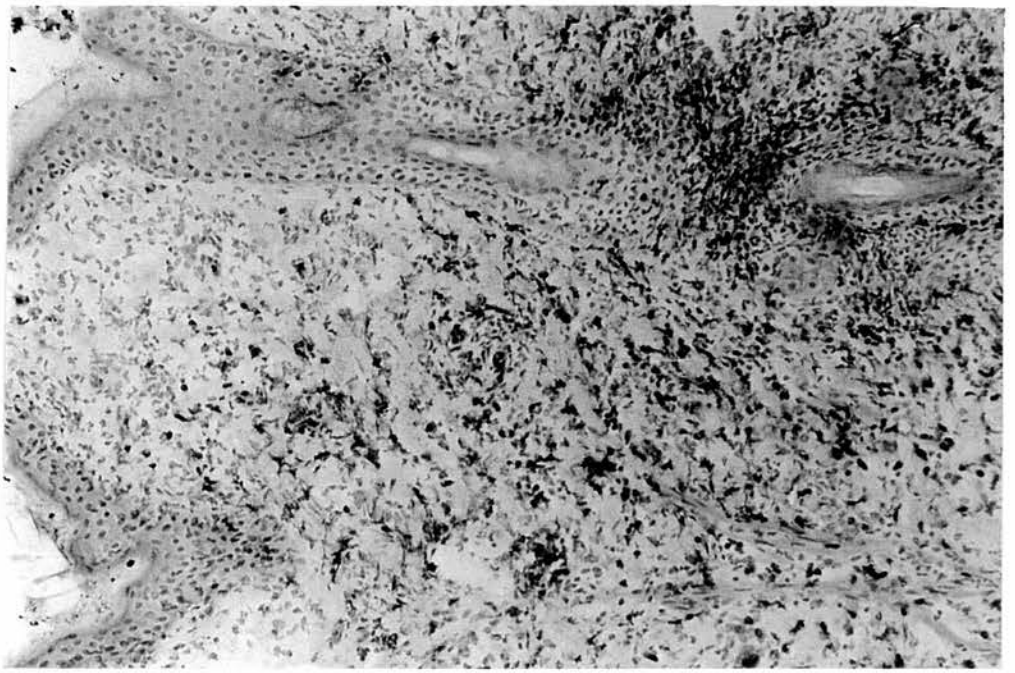


Figure 3.12: Photomicrograph of a 48 hour biopsy of the PPD lesion stained with a monoclonal antibody to $CD4^+$ (SBU-T4). A much greater number of cells are present in the dermis, and a majority of these are positively stained. The cells are concentrated in the periadnexal area (AEC, x250).



3.3.5 $CD8^+$ cell counts

In the antigen injected sites, the kinetics of appearance of $CD8^+$ cells was very similar to those of the $CD4^+$ cells, although the number of $CD8^+$ cells was approximately half that of the $CD4^+$ cells. The $CD8^+$ cell numbers rose slightly at 24 hours, and then markedly to reach maximal levels at 48 hours post challenge. The numbers then remained relatively constant throughout the rest of the time course (Figures 3.13, 3.14, 3.21 and 3.22, Appendix 3.3.5).

The control (PBS injected) biopsies exhibited no significant change in $CD8^+$ cell numbers (Figures 3.21 and 3.22, Appendix 3.3.5).

Figure 3.13: Photomicrograph of a 24 hour biopsy of the PPD lesion stained with a monoclonal antibody to $CD8^+$ (SBU-T8). There are relatively few positively staining cells, and these are concentrated perivascularly. (AEC, x250).

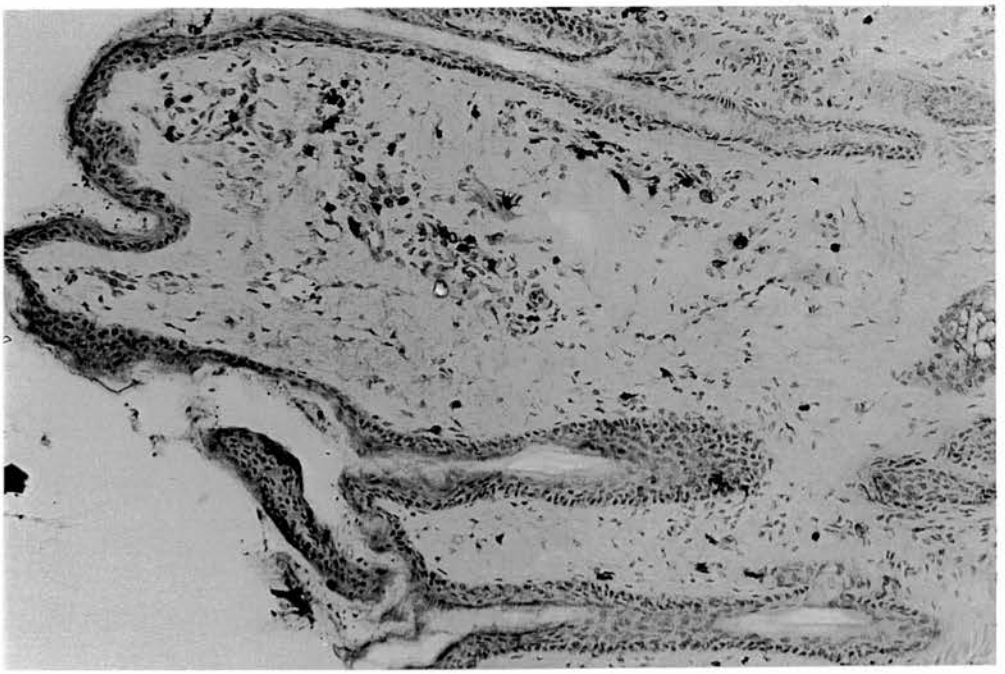
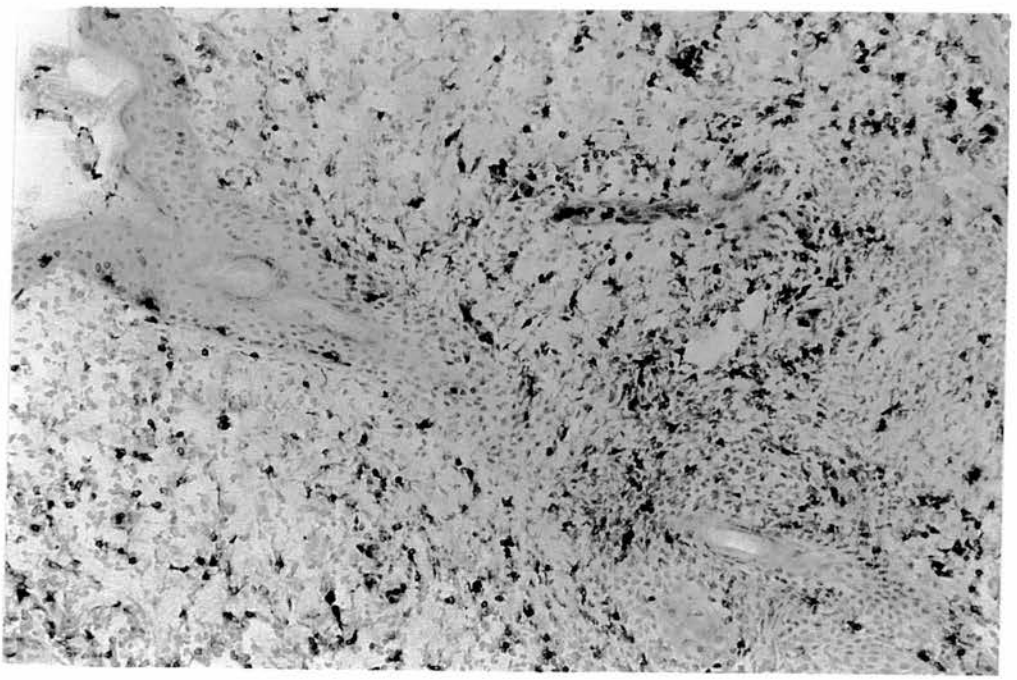


Figure 3.14: Photomicrograph of a 48 hour biopsy of the PPD lesion stained with a monoclonal antibody to $CD8^+$ (SBU-T8). There is an increased number of positively staining cells, and these are concentrated in the periadnexal area. (AEC, x250).



3.3.6 Gamma delta cell counts

Gamma delta T cells were present at relatively low levels in the skin biopsies of both antigen and control (PBS) injected sites. A marginal rise in the gamma delta cells was noted in the biopsies taken from the sites injected with PPD antigen, with levels peaking at 72 hours post injection (Figures 3.23 and 3.24, Appendix 3.3.6).

No alteration in gamma delta cell counts were apparent in the control (PBS injected) biopsy material (Figures 3.23 and 3.24, Appendix 3.3.6).

3.3.7 Macrophage cell counts

Macrophages were present at relatively low levels in the normal skin. In the antigen injected sites, numbers remained static until the 7 hour time point, after which point numbers were reduced markedly to a level at which macrophages were not evident in several sections, with numbers remaining at this low level throughout the time course (Figures 3.15, 3.16, 3.25 and 3.26, Appendix 3.3.7).

The numbers of macrophages counted in the control (PBS injected) biopsies remained relatively constant throughout the time course (Figures 3.15, 3.16, 3.25 and 3.26, Appendix 3.3.7).

Figure 3.15: Photomicrograph of a 24 hour biopsy of the PPD lesion stained with a monoclonal antibody defining macrophages (OM1). There are a small number of cells positively stained in the periadnexal region (AEC, x250).

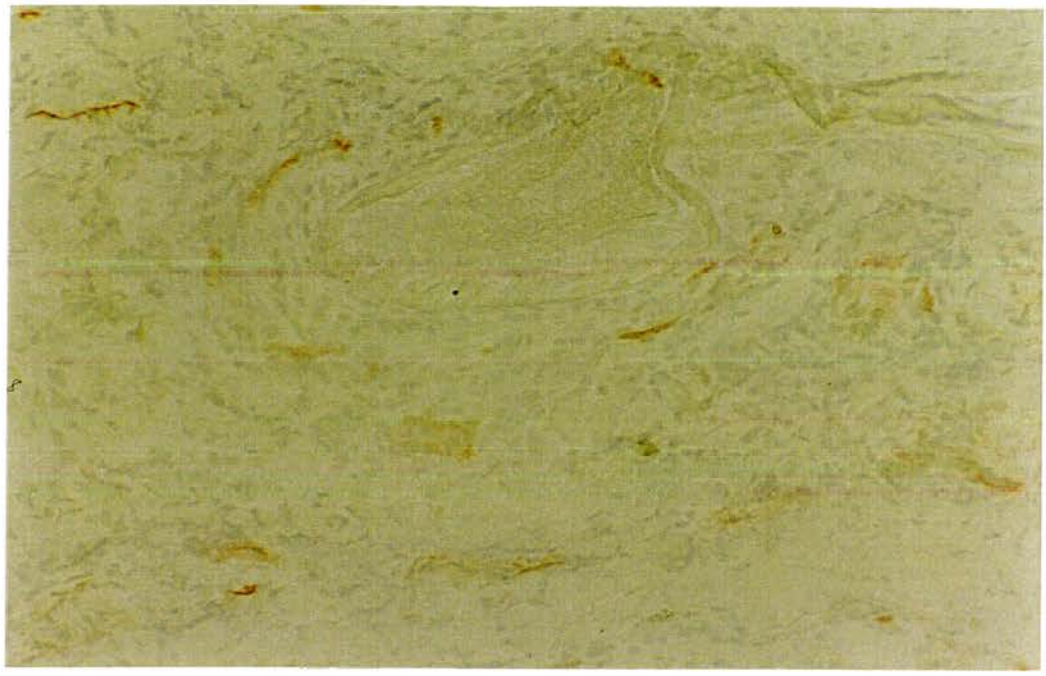
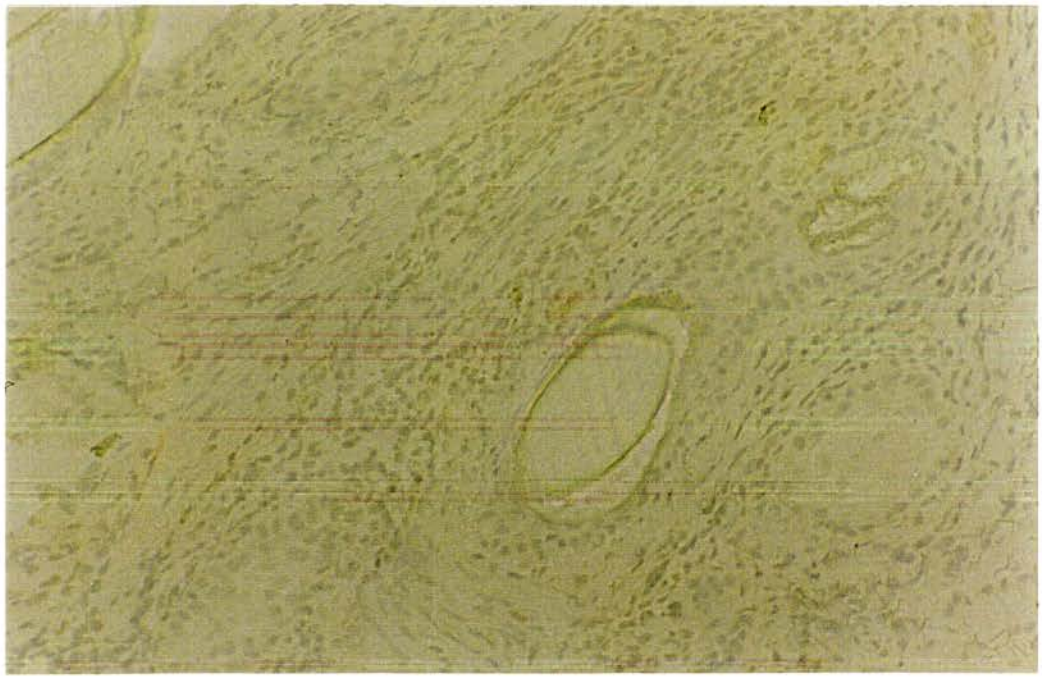


Figure 3.16: Photomicrograph of a 48 hour biopsy of the PPD lesion stained with a monoclonal antibody defining macrophages (OM1). There are no positively stained visible in the section (AEC, x250).



3.3.8 MHC class II expression

The pattern of MHC class II expressing cell counts was very similar to the pattern of macrophage cell counts, with the number of cells expressing MHC class II constituting a relatively low proportion of cells in the reaction. Numbers were similar to control biopsies in the early reaction, and then declined at 24 hours becoming, and remaining, almost negligible by the 48 hour time point (Figures 3.27 and 3.28, Appendix 3.3.8).

There were no apparent alterations in the number of MHC class II expressing cells in the control (PBS injected) biopsies (Figures 3.27 and 3.28, Appendix 3.3.8).

3.3.9 Mast cells and basophils

Mast cells and basophils were only rarely noted in the biopsies examined.

3.3.10 B cells

B cells were only rarely noted in the biopsy material.

Figure 3.17: Comparison of the periadnexal PMN cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)

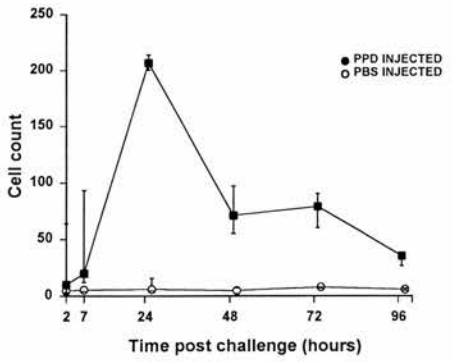


Figure 3.18: Comparison of the dermal PMN cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)

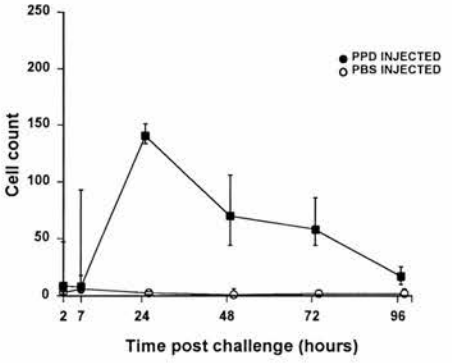


Figure 3.19: Comparison of the periadnexal $CD4^+$ cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)

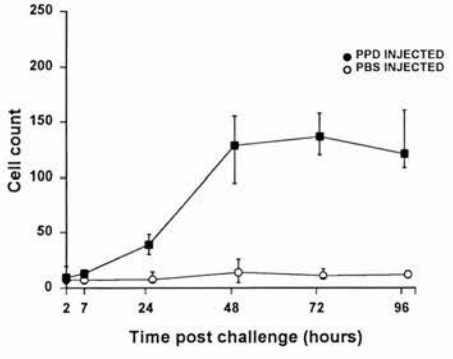


Figure 3.20: Comparison of the dermal $CD4^+$ cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)

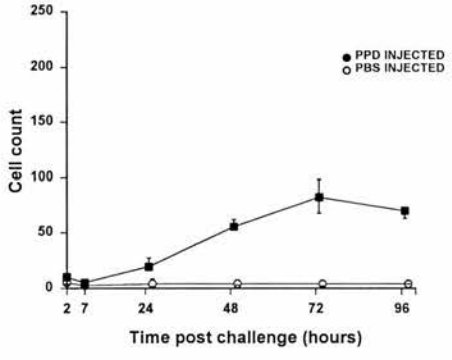


Figure 3.21: Comparison of the periadnexal $CD8^+$ cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)

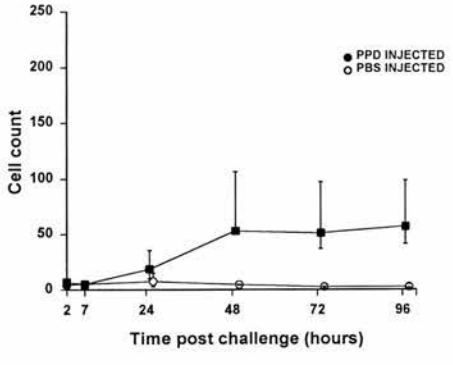


Figure 3.22: Comparison of the dermal $CD8^+$ cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)

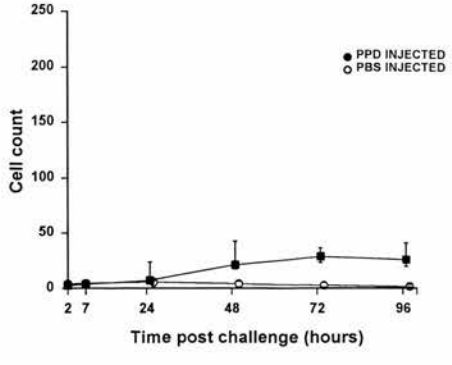


Figure 3.23: Comparison of the periadnexal γδ cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)

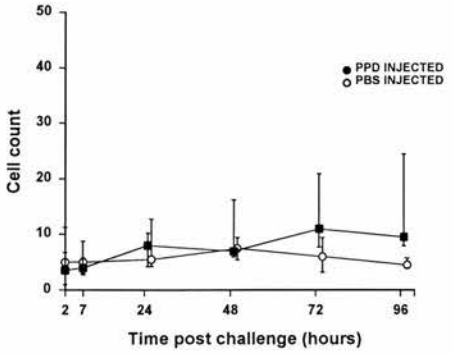


Figure 3.24: Comparison of the dermal γδ cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)

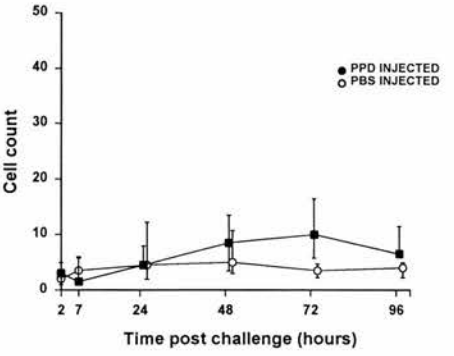


Figure 3.25: Comparison of the periadnexal $OM1^+$ cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)

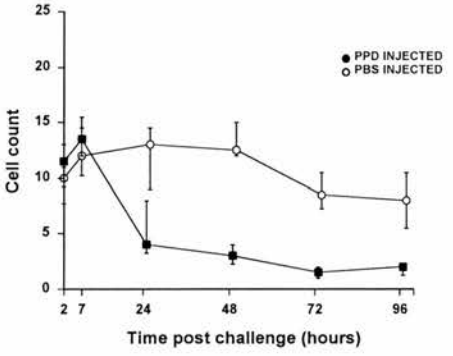


Figure 3.26: Comparison of the dermal $OM1^+$ cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)

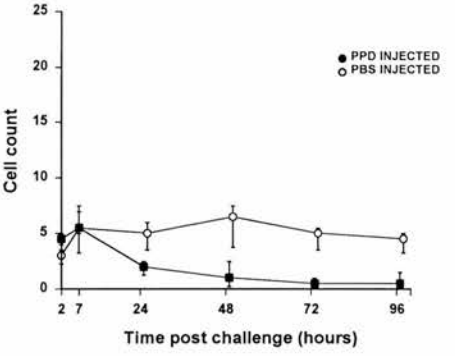


Figure 3.27: Comparison of the periadnexal SW73.2 cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)

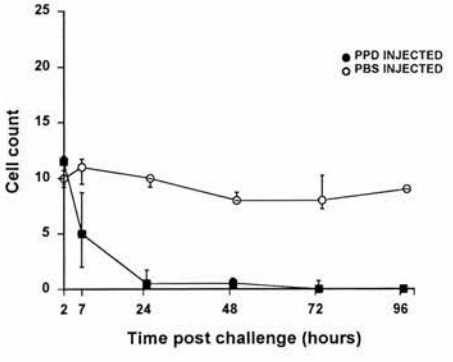
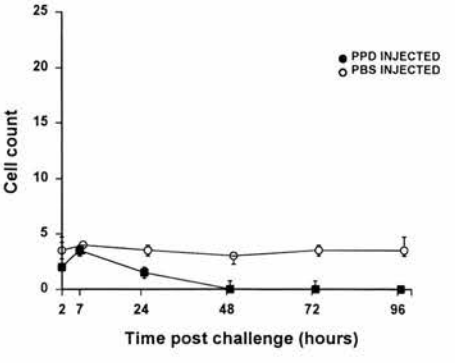


Figure 3.28: Comparison of the dermal SW73.2 cell count following i/d injection of PPD and PBS in sensitised sheep (median and interquartile range indicated)



3.4 DISCUSSION

The gross appearance of this particular sheep DTH reaction, consisting of an indurative plaque of maximal size at 48-72 hours post challenge, is consistent with that described in other species. Histopathologically, the cell type found to predominate in the early reaction was the PMN. Previous studies have provided controversy as to whether PMNs are present at all in DTH lesions, with some workers reporting a high number of these cells in the early reaction (Feldman, Fitch, 1937; Long, 1937; Follis, 1940; Dienes, Mallory, 1932; Gell, Hinde, 1951; Boughton, Spector, 1963; Legendre et al. 1979; Platt et al. 1983; Norris et al. 1991), and many others failing to note the presence of PMNs at all. Many authors that describe the presence of PMNs in the early lesion have considered them to be a non specific reaction to tissue damage, and not a specific component of the DTH (Dienes, Mallory, 1932; Gell, Hinde, 1951; Boughton, Spector, 1963). A recent study, concentrating specifically on PMN migration into DTH lesions, indicated that these cells were not present in a dermal DTH reaction, although this study only evaluated biopsies at post 24 hour time points when it would appear that the PMN has become less predominant in the lesion (Gao et al. 1994). The active contribution of the PMN to the DTH response has been evaluated via the use of PMN depleting monoclonal antibodies in the rat model (Kudo et al. 1993a; Kudo et al. 1993b), where it has been shown that the depletion of PMNs at either the priming or initiation phase of a DTH type model resulted in the markedly reduced size of the DTH and lowering of mononuclear cell infiltration into the lesion.

The consistent finding of a relatively large number of PMNs in the early lesion in this study would suggest that they have an important role to play in the development of a DTH, presumably through the release of one or several of the proinflammatory mediators which they contain. There is some evidence indicating that PMN degranulation is indeed involved in the swelling associated with a DTH like

response, with the level of degranulation controlled by the lymphocytes in the reaction. There may also be a possibility that the PMNs are attracted to the site by a mechanism that is not antigen specific, and it would have been of value to investigate the histopathological characteristics of a PPD injection site in a non sensitised animal.

Another interesting finding of this work is the relatively low levels of macrophages found in the reaction at the later time points. A majority of the previously published work has not defined macrophages using monoclonal antibodies, and in fact in most instances they are defined purely on their morphological appearance or loosely grouped as lymphohistiocytic cells. Several studies utilising monoclonal antibodies to study the kinetics of macrophage appearance have indicated a considerable presence in the later lesion (Poulter et al. 1982; Konttinen et al. 1983; Platt et al. 1983) This has led to a situation where macrophages are commonly considered as part of the DTH reaction. Only one previous report, using histochemical markers for differentiating the macrophage, has indicated that macrophages decline in numbers in the later lesion (Turk et al. 1966). This led to the consideration in this study that the particular macrophage cellular marker against which the monoclonal antibody was directed, was lost upon cell activation, and hence two other monoclonal antibodies (VPM63 and VPM65) were used to stain representative biopsy samples. The staining pattern was similar to that obtained with the OM1 monoclonal antibody. The role of the macrophage in the initiation of the response has been previously indicated (Torii et al. 1993), and their low levels in the later reaction would suggest that they have trafficked away from the lesion. The most likely pathway for this traffic would be to the local lymph node via the lymphatics, where a subsequent immune reaction to the antigen could be stimulated. Indeed a study of the lymph node draining the site of a DTH in the cat (Legendre et al. 1979), indicated a strong macrophage response, and, although the source of the macrophages was not investigated, it would seem likely that they originated from the DTH reaction.

The relatively large contribution of the $CD4^+$ and $CD8^+$ cells was expected, as these cells constitute a major part of the cell mediated immune system, and have previously been consistently reported as being involved in the DTH response (Poulter et al. 1982; Konttinen et al. 1983; Platt et al. 1983; Gibbs et al. 1984). Their presence in a ratio roughly equivalent to that reportedly found in peripheral blood (Lujan, 1994) would suggest that the attraction of lymphocytes into the lesion is not selective to one individual subtype. The attraction of these lymphocytes may be directed by a range of chemoattractants, and it could be speculated that these have been released, at least in part, by the PMNs present in the earlier lesion.

The contribution of gamma-delta cells to the classical DTH lesion has not been previously reported, although one study of a similar contact allergy type DTH reaction in human patients indicated that these cells were involved in the resolution rather than the initiation of the reaction (Fujita et al. 1993). The facts, however, that gamma-delta cells constitute a much larger proportion of circulating lymphocytes in sheep compared to man (Hein, Mackay, 1991),and that gamma-delta cells have been reported to be closely associated with immune responses to mycobacteria (Janis et al. 1989), suggested that they may have an important role in the ovine tuberculin DTH reaction. The results of this study indicate that gamma-delta cells do not migrate into the DTH lesion in any significant numbers, and suggests that they do not contribute to the development of the lesion. The circulating levels of gamma-delta cells in sheep do however decline with increasing age, which may have contributed to the findings.

The relatively low numbers of mast cells and basophils is in accordance with the work of Torii et al (1993), who indicated that the classical tuberculin response was macrophage dependent and not reliant on mast cells or basophils.

The presence of marked levels of fibrin and oedema fluid in the reaction was expected, and was largely responsible for the induration associated with the reaction.

The absence of B cells was expected in a reaction that is associated with a cell mediated immune response, and classically independent of the humoral immune system.

In conclusion, this work has outlined the relative contributions of the major immune cell types to the classical tuberculin reaction in the sheep. The significant presence of the PMN in the early lesion has been shown, this clarifying the previously disputed role of this cell in the DTH reaction. The disappearance of the macrophage in the later lesion again clarifies an area of previous dispute, and suggests that these cells migrate out of the later lesion, most probably to the local regional lymph node. The low levels of gamma-delta cells in the DTH reaction has been shown, suggesting that these cells have a relatively low contribution to the reaction. The high levels of $CD4^+$ and $CD8^+$ lymphocytes confirms their importance in this reaction, as would be expected in a cell mediated immune response. A study of the levels of cell replication occurring in these lymphocyte populations utilising a proliferation marker such as PCNA, would have been of interest, reflecting the degree of activation and antigen specificity of these cell populations.

Subsequent to the submission of this thesis, a similar study of the bovine tuberculin reaction has been published, with results largely in accordance with the above findings. The notable differences are in the findings that the bovine DTH response has an initial significant presence of gamma-delta T cells and a subsequent significant presence of macrophages. The difference in gamma-delta response may be related to the age of the animals as discussed earlier, although the authors only indicate the age of half the animals studied. The difference in macrophage counts may reflect a real species difference, or may be related to the monoclonal antibody used in the bovine study, which was not specific for the macrophage (Doherty et al. 1996).

CHAPTER FOUR

INVESTIGATION OF THE GROSS CHARACTERISTICS OF THE DTH IN SHEEP INFECTED WITH MVV AND ITS RELATIONSHIP WITH LUNG PATHOLOGY

4.1 INTRODUCTION

MVV is the prototype (ovine) lentivirus being closely related to the lentiviruses infecting other species, most notably HIV (Gonda et al. 1985). MVV differs from most related viruses in that infection is not associated with clinically recognised immunosuppression or clear patterns of secondary opportunistic infection, although some workers have suggested an increased susceptibility to certain ovine lung pathogens (Myer et al. 1988; Giangaspero et al. 1993). This is suprising and contrasts with the well recognised progressive immunosuppression and pattern of secondary infection documented in HIV infection (Redfield et al. 1986), associated with a declining capacity of the cell mediated immune system to respond to pathogens. This different pattern of clinical infection may result from the fact that MVV primarily infects cells of the monocyte/macrophage series (Gorrell et al. 1992; Lujan et al. 1994), whereas HIV also infects $CD4^+$ cells, resulting in a declining number of these cells in the later stages of the disease process (Redfield et al. 1986; Embretson et al. 1993).

The DTH response involves the interaction of antigen presenting cells with antigen specific T lymphocytes (McKeever, 1994). It is commonly utilised to test the *in vivo* activity of the cell mediated immune system, and has been shown to provide an accurate indication of the capacity of this arm of the immune system to respond to

an *in vivo* antigen challenge (Otto et al. 1993). It has been used extensively in the evaluation of the immunosuppression produced by HIV infection, and is a component of the standard Walter Reed staging classification of the progression of HIV infection to AIDS (Redfield et al. 1986). The use of the DTH as an assessment of the *in vivo* cell mediated immune response in sheep infected with MVV has been reported previously by two groups, both of which studied the response in sheep experimentally infected with the virus and at an early time point after infection. The first study (Larsen et al. 1982b), found no difference in the size of the DTH response in the sheep infected with MVV when compared to a control group, although both groups showed variable responses. The second study (Myer et al. 1988), concentrated on the experimental coinfection of lambs with MVV and SPA virus, and provided limited evidence that MVV infection alone resulted in a decline in the DTH response. This latter study remains the only documented evidence for altered *in vivo* immune responses in sheep infected with MVV.

In HIV infection, the degree of depression of the DTH has been correlated with the progression of the infection to AIDS (Redfield et al. 1986) and the decline in number of $CD4^+$ cells (Borleffs et al. 1991; Blatt et al. 1993). There is no equivalent progression to a recognised immunodeficiency stage or decline in $CD4^+$ cell numbers (Lujan et al. 1993) in MVV infection, and the main assessment of the stage of disease progression in infected individuals is the post mortem investigation of the degree of pathological change present in target organs. This pathological change has been shown to be associated with the expression of viral protein (Brodie et al. 1995), and the degree of pathological change is therefore correlated with the level of viral replication and viral protein expression in the individual.

This study was undertaken to investigate the DTH response in sheep naturally infected with MVV, in particular sheep which had been seropositive for several years, in order to clarify whether there is a decline in DTH response in sheep naturally infected with MVV; and, further, to investigate if there was any link between the strength of the DTH and the level of viral multiplication as assessed by the degree of classical MVV associated pathological changes in the lung.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Five adult female sheep were used in this study (Table 4.1). They were selected from the flock of naturally infected sheep maintained at the University of Edinburgh Veterinary School (2.1). The sheep had all been serologically positive for MVV infection for a considerable period of time (Table 4.1), as defined using the AGIDT described previously (2.2).

Table 4.1: Details of the sheep used in this experiment

Sheep Number	Breed	Age (years)	Sex	Years of seropositivity
004	Texel	4	F	2
10	Texel	9	F	4
20	Texel	7	F	4
27	Bleu de Maine	5	F	4
57	Texel	9	F	4

4.2.2 DTH initiation and measurement

The DTH was initiated and measured in the standard manner described in 2.4 - 2.5.

4.2.3 Post mortem technique and lung sample preparation

At the conclusion of the of the DTH experiment, the sheep were euthanased and examined at post mortem in the manner described in 2.10.1, and the lung samples selected and prepared as described in 2.10.2. The lung specimens obtained from these MVV infected sheep were pooled with identically treated specimens obtained from the clinically healthy sheep used in the previous experiment (Chapter 3).

4.2.4 Lung pathology assessment

The four sections (from both the MVV infected and clinically healthy sheep) were assessed by two independent pathologists in an observer blinded subjective assessment (2.10.3). The total score of each parameter for the four sections was recorded and utilised for statistical evaluation.

4.3 RESULTS

4.3.1 Skin thickness

The gross reactions in the MVV infected sheep were similar in appearance to those produced in the control animals, consisting of a raised, reddened indurative plaque, which was maximal at 48-72 hours post injection. There was, however, a marked variability in the increase in skin thickness recorded in the different sheep (Figure 4.1, Appendix 4.3.1), with a significant depression in the size of the reaction at 24, 72 and 96 hours post injection when compared to the values obtained from the clinically healthy sheep in the previous chapter (Figure 4.2).

Figure 4.1

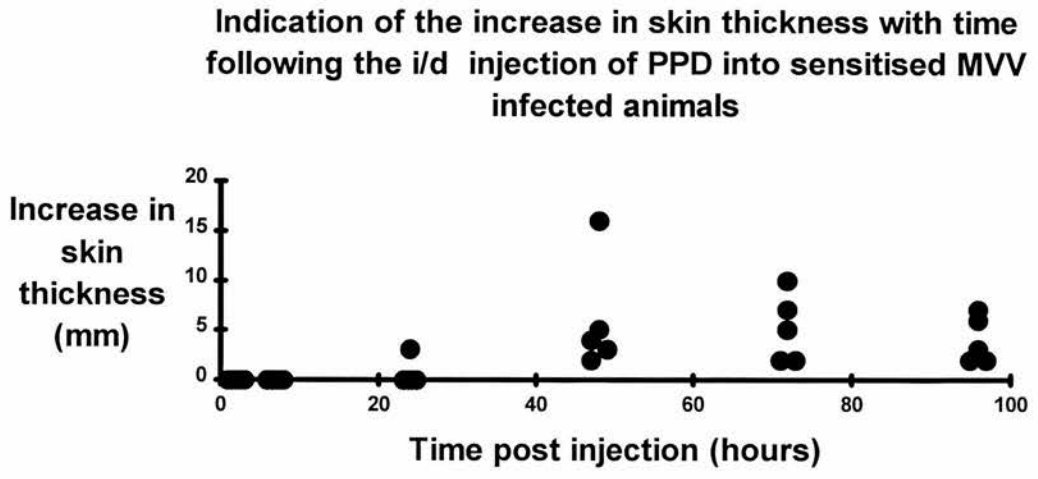
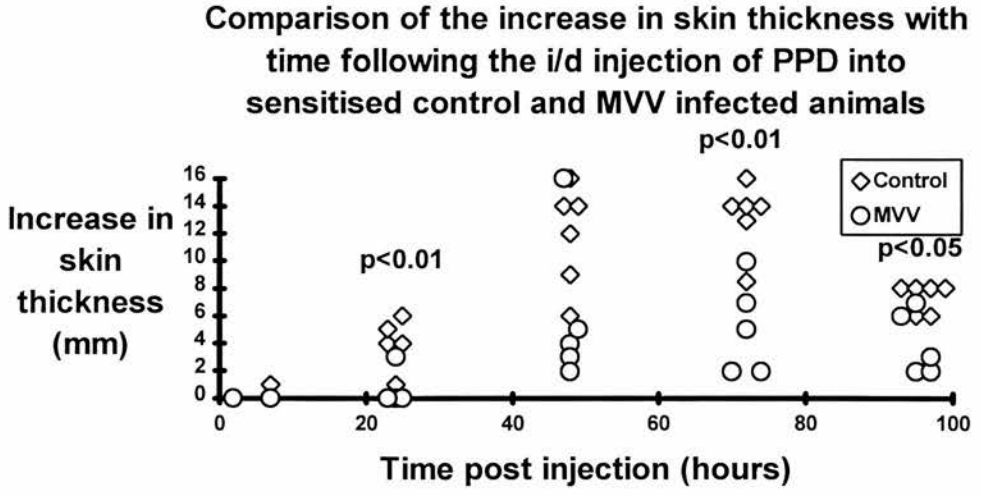


Figure 4.2



4.3.2 Lung pathology quantification

The numerical values obtained from the subjective assessment of lung pathology by the two independent observers were not identical. When the values were ranked, however, there was a very strong correlation between the two assessments for all the parameters graded (Table 4.2, Figures 4.3-4.5, Appendix 4.3.2). Classical Maedi lesions were present in several sheep, consisting of interstitial pneumonitis, smooth muscle hyperplasia, and lymphoid follicle development (Figures 4.6 and 4.7). There were no significant differences between the MVV and control animals in terms of the parameter scores assessed by either pathologist, suggesting that most of the MVV group, although seropositive, were not suffering from advanced Maedi lesions at the time of euthanasia.

Table 4.2: Table indicating the degree of correlation between the lung parameter assessments of the two independent pathologists (Spearman correlation statistic)

	Parameter assessed		
	Interstitial reaction	**Degree of smooth muscle hyperplasia**	**Number of follicular structures**
Correlation between the independent scores for these parameters	$r_s = 0.906$ $p < 0.0005$	$r_s = 0.602$ $p < 0.05$	$r_s = 0.818$ $p<0.0025$

Figure 4.3

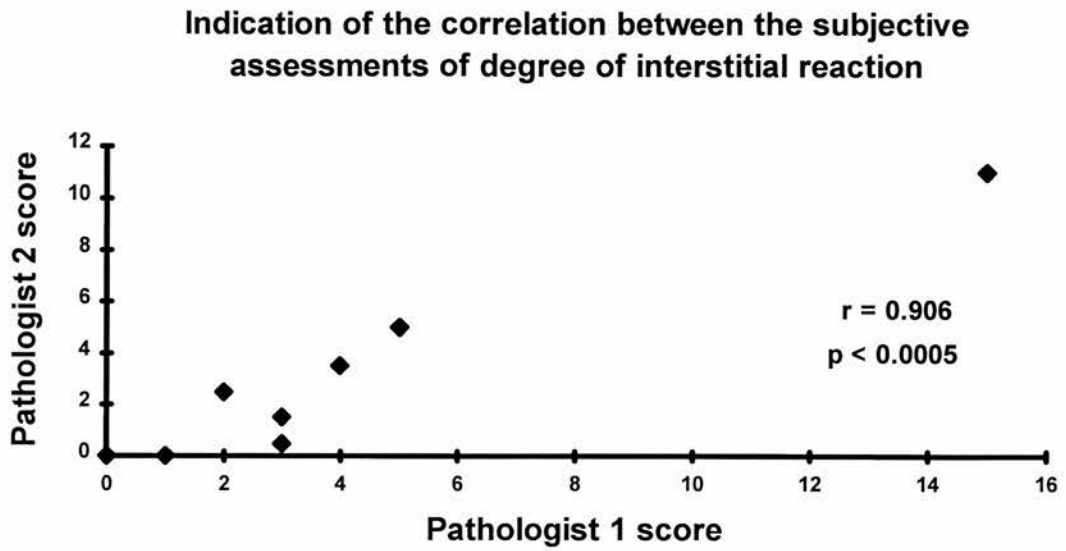


Figure 4.4

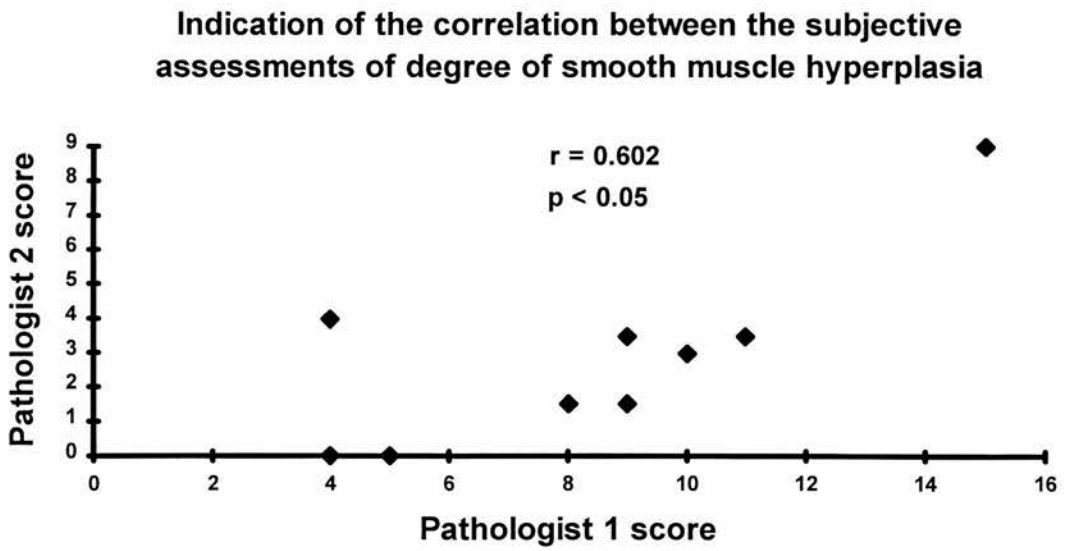
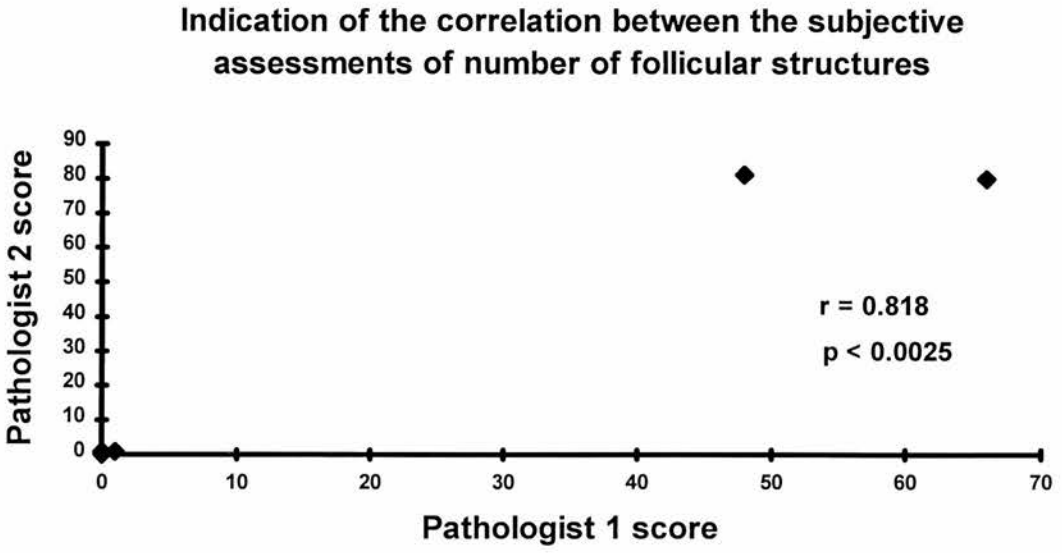


Figure 4.5



4.3.4 Correlation between DTH response and degree of lung pathology

There were no significant correlations found between the scores of the three parameters of lung pathology indicated by either pathologist, and the size of the gross DTH response (Appendix 4.3.4).

Figure 4.6: Photomicrograph of a representative lung section from sheep exhibiting the classical features of Maedi pathology: interstitial pneumonitis, smooth muscle hyperplasia, and lymphoid follicle presence. Several alveoli contain activated macrophages (H&E, x40).

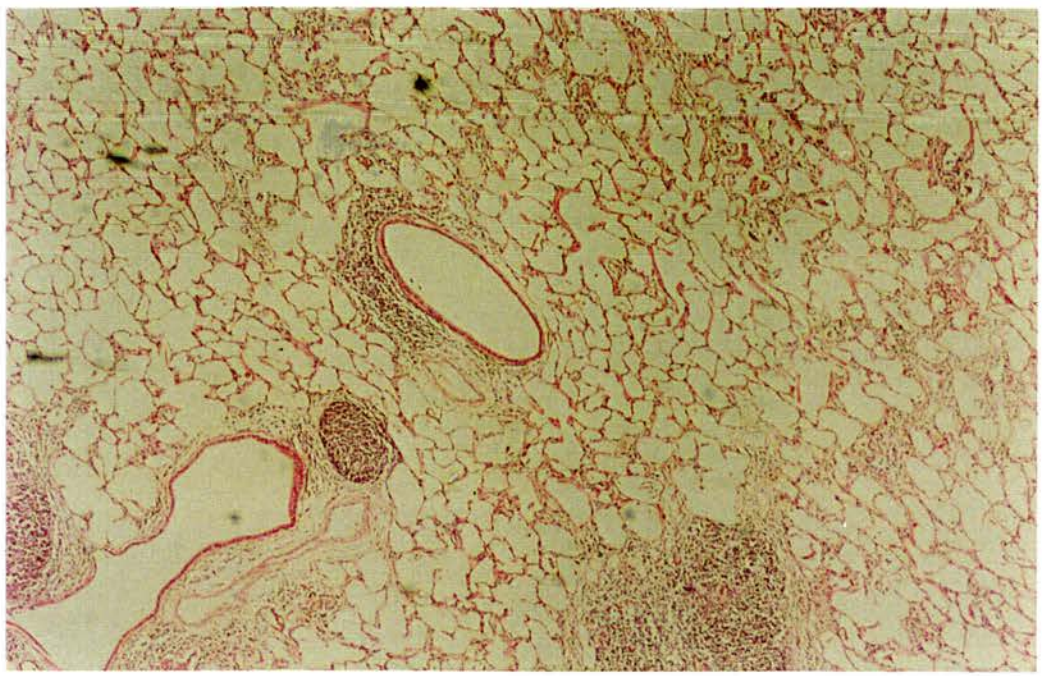
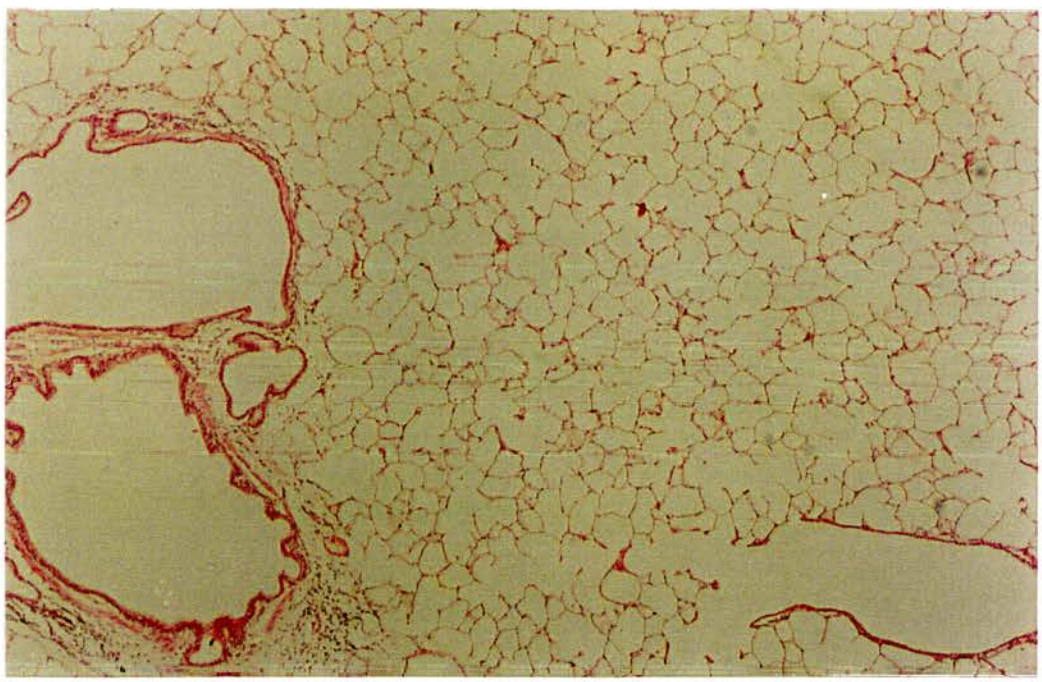


Figure 4.7: Photomicrograph of a section of lung taken from a control sheep in an anatomically matched area to the above figure 4.6. Note the complete absence of any inflammatory features or smooth muscle hyperplasia (H&E, x40)



4.4 DISCUSSION

This study has shown that a majority of sheep naturally exposed, and seropositive, to MVV exhibit grossly depressed DTH responses to PPD. There is no previously published investigation of the gross DTH in sheep naturally infected with MVV, although these results concur with the previous work of Myer et al (1988), who indicated a depression in DTH response size in sheep experimentally infected with MVV. Such a depression in DTH size was not indicated in a similar experimental infection study by Larsen et al (1982), although response size in both the infected and control groups was variable. The finding of a depression in DTH response size correlates with the well documented decrease in DTH response size seen in the latter stages of HIV infection, considered indicative of progression to AIDS (Redfield et al. 1986; Colebunders et al. 1989; Borleffs et al. 1991; Blatt et al. 1993; Birx et al. 1993; Pesanti, 1994), and a similar decrease in DTH response size that has been reported in FIV infection (Otto et al. 1993). There is no previously published study indicating the mechanisms responsible the depression of the DTH in lentivirally infected individuals, although in HIV infection the relative size of the DTH has been shown to correlate with the decline in $CD4^+$ cell numbers that occurs in the latter stages of the clinical disease (Borleffs et al. 1991; Blatt et al. 1993). Such a decline in $CD4^+$ cells is not associated with MVV infection(Lujan et al. 1993), and therefore is not available as an explanation for the depressed DTH response.

The association of the depression in DTH response with the advanced stage of disease is well established in HIV infection (Redfield et al. 1986; Colebunders et al. 1989; Borleffs et al. 1991; Blatt et al. 1993; Birx et al. 1993; Pesanti, 1994), and has been indicated in FIV infection (Otto et al. 1993). The assessment of stage of disease progression is more difficult in MVV infection as there is no recognised pattern of secondary infection or recognised change in circulating $CD4^+$ cell numbers. In this

study, this assessment of stage of disease progression was made using a subjective quantification of the degree of classical MVV pathology, this having been shown previously to be associated with the level of viral protein expression and replication (Brodie et al. 1995). There was no association between the scores of lung pathology and the degree of depression of the DTH response, suggesting that the alteration in DTH responsiveness is independent of the viral load in the host. This finding is in agreement with an extensive study of the depression of DTH response in HIV infected people, which investigated the depressed DTH response in conjunction with an assessment of proviral DNA load, lymphocyte abnormality, and antiviral antibody status indicated that the suppression in DTH response was associated with HTLV-1 infection *per se*, and not with viral replication or load (Welles et al. 1994). This result is surprising considering the reported association between the depression of DTH response and progression of HIV infection to AIDS, although the study by Welles et al (1994) also reported that the depression of DTH was associated with changes in the number of lymphocytes and monocytes, and hypothesised that the depression of DTH was caused by altered cytokine production in viral carriers that leads to an alteration in the cell populations involved in the DTH.

In conclusion, this study has shown that sheep naturally exposed to and seropositive for MVV have significantly depressed DTH reactions when compared to uninfected control sheep. This depression in DTH response size is independent of the degree of classical MVV-associated pathology in the lungs of the infected sheep, and may therefore be independent of the viral load in the infected animals. It would have been interesting to actually assess the level of viral load in these animals using molecular biology techniques.

CHAPTER FIVE

IMMUNOHISTOLOGICAL ANALYSIS OF THE DEPRESSED DTH IN MVV INFECTED SHEEP

5.1 INTRODUCTION

The previous chapter has described the finding of a depressed DTH response in sheep that were seropositive for MVV following natural exposure to the virus, and indicated that the degree of depression of the DTH is variable. Furthermore, it has shown that the degree of depression of the DTH is not associated with the degree of pathological change in the lung.

In order to investigate this deficiency in development of the DTH further, there is a need to characterise the immunohistological nature of the MVV associated reduction in DTH response size, relating the decreased MVV response to that of clinically normal control animals. The previous study of the normal ovine DTH response (Chapter 3), indicated that the reaction was maximal at 48-72 hours post injection, and was characterised by two main types of cellular infiltration: an initial PMN infiltrate at 24 hours, and a subsequent $CD4^+/CD8^+$ influx at 48-72 hours post PPD injection. The time points of 24 and 72 hours post injection were therefore selected for evaluation of the MVV response in comparison to similar control biopsies, with a full range of cell types being investigated using histochemical and immunohistochemical techniques. There have been no reported equivalent studies performed in HIV infected humans, although the depression in DTH associated with HIV infection is a well recognised phenomenon.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Six adult female Texel sheep were selected from the MVV infected flock (2.1, Table 5.1). All of these sheep had exhibited a persistent serological response to MVV, as defined using a standard agar gel immunodiffusion test (2.2, Appendix 5.2.1). Clinical examination, particularly of the respiratory and nervous systems, of these sheep revealed no detectable abnormality, and routine haematological samples revealed a mild neutrophilia in sheep 076 to be the sole abnormal finding (Appendix 5.2.1). Five sex and breed matched control sheep of similar age to the infected ewes were obtained from accredited flocks (2.1, Table 5.1). These control sheep were clinically and haematologically normal, and serologically free from MVV infection at the time of challenge (Appendix 5.2.1).

Table 5.1

Sheep Number	Status	Breed	Age (years)	Sex
011	MVV +ve	Texel	9	Female
025	MVV +ve	Texel	9	Female
035	MVV +ve	Texel	8	Female
071	MVV +ve	Texel	9	Female
076	MVV +ve	Texel	8	Female
118	MVV +ve	Texel	8	Female
183	Control	Texel	8	Female
184	Control	Texel	9	Female
245	Control	Texel	8	Female
246	Control	Texel	7	Female
247	Control	Texel	9	Female

5.2.2 Blood sampling

Blood was collected from the sheep at the time of initiation of the DTH and analysed for white blood cell parameters as described previously (2.3).

5.2.3 DTH initiation, measurement, and biopsy

The DTH was initiated, measured, and biopsied in the standard manner described in 2.4 - 2.6. In this particular experiment, biopsies were taken at 0 hours, 24 hours and 72 hours post injection, these being the time points which the investigation of the normal DTH had shown to be time points associated with the significant infiltration of the lesion with PMNs and $CD4^+$ cells respectively.

5.2.4 Immunohistopathological staining

Routine paraffin embedded sections were processed and stained with haematoxylin and eosin as described in chapter 2.

Frozen sections were processed as described in chapter 2 and stained with monoclonal antibodies SBU-T4 ($CD4^+$ T cells), SBU-T8 ($CD8^+$ T cells), 86D (gamma delta T cells), DU2.87 (B cells), VPM63 (macrophages), VPM 65 (macrophages) and OM-1 (macrophages) (chapter 2).

5.2.5 Quantification

An initial subjective appraisal of the sections revealed that the reactions in both groups exhibited very similar patterns of cellular infiltration, consistent with the pattern of infiltration found in the study of the normal DTH reaction (3.2.4). As before the periadnexal structures and superficial dermal areas of the histological sections were noted as areas of cellular accumulation, and these areas were once again selected for cell counting.

The method utilised for counting the cellular infiltrate in Chapter 3 was an adaptation of the most comprehensive previously published method for the evaluation of cellular infiltrates in the skin, with a separation of the counting into the distinct areas of cellular accumulation. After careful consideration of this method of counting, the author considered that there was a potential for error and subjective interference in the method of counting the periadnexal regions, with the selection of grid squares that were considered to be periadnexal being reliant upon the subjective appraisal of the observer. In order to rectify this problem, use was made of a computer assisted image analysis system (Quantimet 500C image analysis system, Leica Instruments, Cambridge, UK), which allowed standardisation between different sections, eliminating the possibility of subjective operator interference and therefore maximising the accuracy of the cell counting. The method was developed and then stored in the computer as a specific routine that could then used on every section counted (Appendix 5.2.4). The routine followed a similar pattern to the previous manual method of counting, beginning with the periadnexal area count. To begin with, the adnexal structure of interest was first outlined manually using the cursor. The computer then expanded this outline by a specific distance which had been judged to be the area of accumulation of cells around the adnexae, and produced a binary image counting window in which the cells of interest were then manually selected

using the cursor. The computer then counted the number of cells indicated, and measured the area of the counting window, producing a figure for the number of positive cells per mm^2 of tissue section. An initial pilot study of several sections was undertaken to ascertain the reproducibility of individual counts and variability between different sections in order to ascertain the number of counting fields required to produce an average final figure that was a true representation of the cell density in each specimen (Aherne, Dunnill, 1982). This study indicated that this computerised method produced a consistent result within each area counted, and also that there was relatively little variation between different fields on an individual section. The number of fields to be counted for each section was decided by evaluating the number of fields required to produce a result whereby the standard error of the counts was less than 5% of the their mean, which may be considered as a statistically valid representation of the true figure (Aherne, Dunnill, 1982), the mean being the figure that would be recorded as the result. This trial indicated that an evaluation of six individual fields would satisfy this statistical requirement, and this number of repetitions was included in the counting routine. In the case of the superficial dermal area, the area to be counted was manually outlined to avoid extraneous structures such as hair follicles. The computer then outlined the area with a binary overlay image, and produced a counting area window. The cells of interest were indicated manually, and the computer produced a figure of cells per mm^2. As above, a trial was undertaken to ascertain the number of fields required to be examined in order to produce a statistically significant result, and a figure of six fields was produced. This number of fields was incorporated into the computer routine.

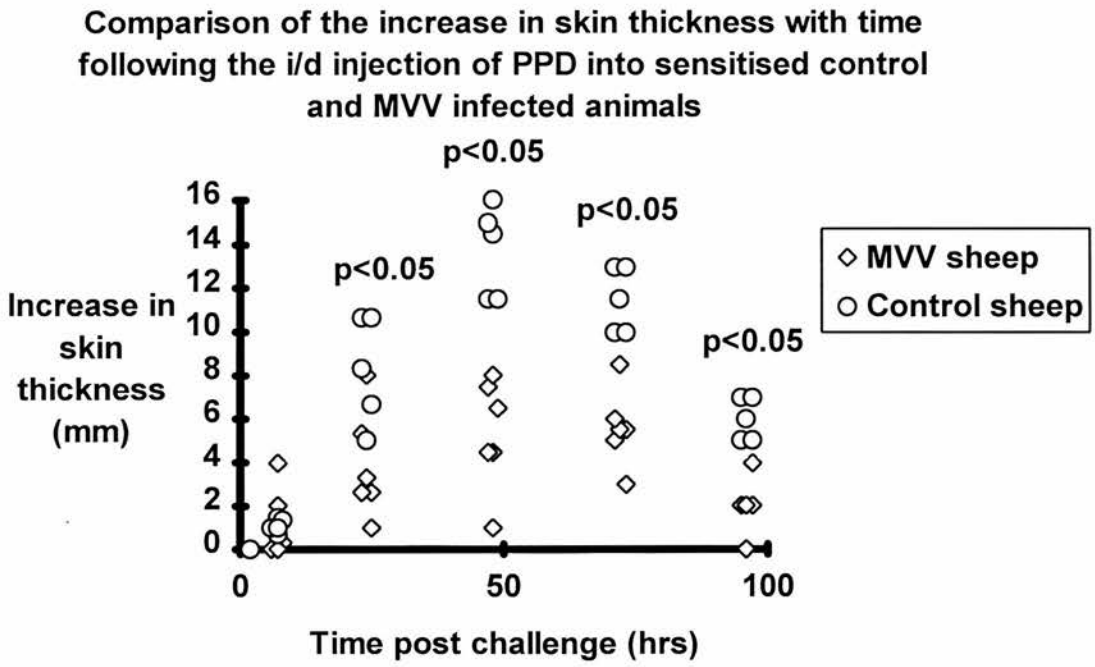
All sections were evaluated for cell counts using this computer system.

5.3 RESULTS

5.3.1 Skin thickness

MVV infected animals had significantly lower increases in skin thickness compared to the control animals at all measurement points after 7 hours (Figure 5.1, Appendix 5.3.1), the significance being $p<0.05$ using a Mann-Whitney test. There was, however, a marked variation in results within the MVV group. No change in skin thickness was seen in the control PBS injected sites.

Figure 5.1



5.3.2 Histopathological assessment

Within the control group of animals, the histological characteristics of the reaction were indistinguishable from those of the previous control animals (3.3.2), consisting of a predominantly PMN infiltrate at 24 hours post injection followed by a predominantly mononuclear cell infiltrate at 72 hours post injection. The biopsies from the MVV infected sheep exhibited a subjectively lower level of cellular infiltrate into the lesion at the 24 hour time point, with a subjectively similar reaction at the 72 hour time point.

The control biopsies of both groups exhibited no apparent change from the normal skin.

5.3.3 Polymorphonuclear neutrophils

PMNs were relatively sparse in normal skin of both groups, but were the predominant infiltrating cell in the PPD induced lesion at the 24 hour time point biopsy (Appendix 5.3.3). There was a significant depression in the PMN density in the MVV infected sheep ($p<0.01$) at this time point (Figures 5.2, 5.4 and 5.5). MVV lesions exhibited a marked variability in the numbers of cells in the lesion, contrasting with the consistent levels found in the control animal reactions. Highly significant correlations between the numbers of cells in the periadnexal area in the 24 hour biopsy and the eventual thickness of the gross lesion (Figure 5.3, Table 1) were present. By the 72 hour time point, PMNs were present at reduced densities and there were no significant differences between the groups. There were no significant alterations in the PMN numbers in the control biopsies.

Figure 5.2

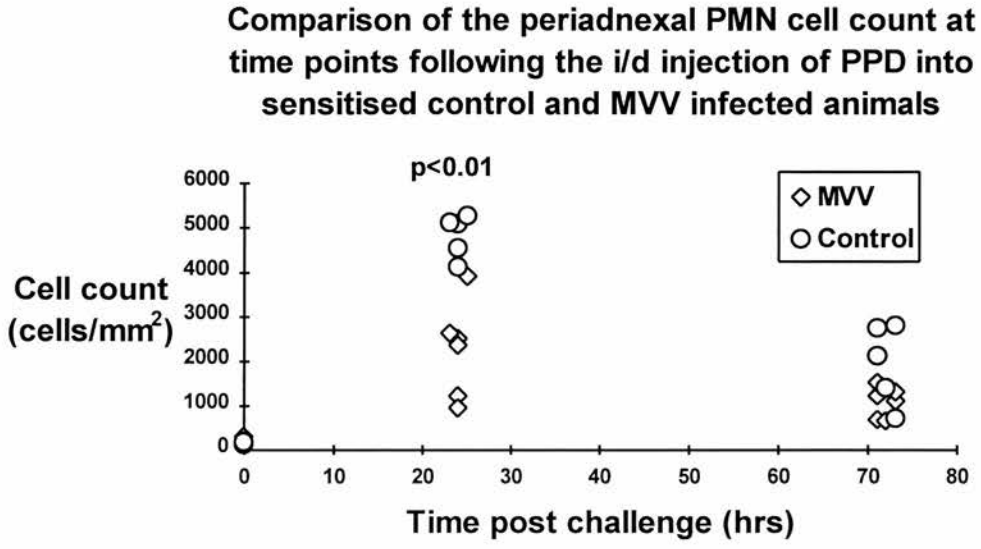


Figure 5.3

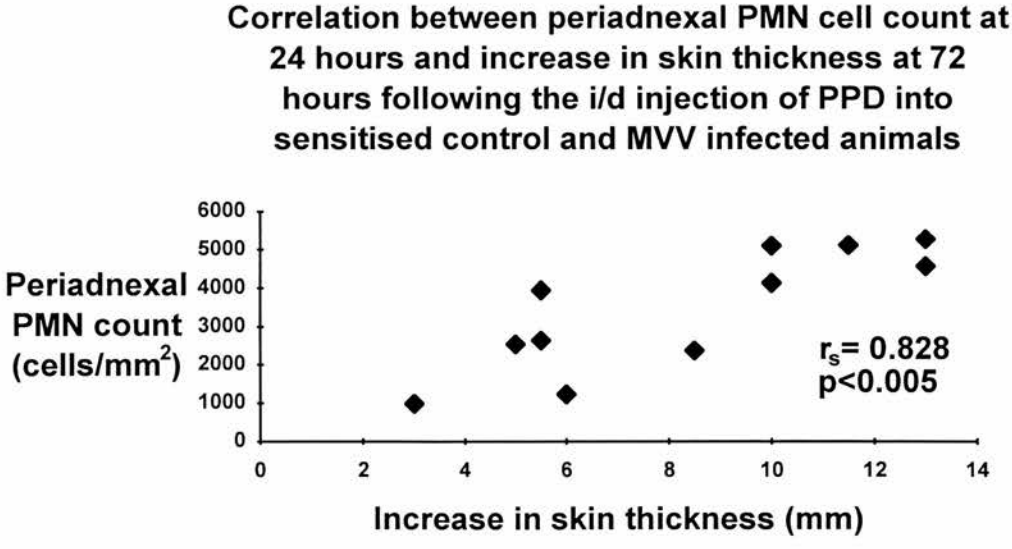


Table 5.2 Correlations between the periadnexal 24 hour cell count of PMNs and the increase in skin thicknesses at 48, 72 and 96 hours post the i/d injection of PPD into sensitised control and MVV infected animals (Spearmann ranking correlation statistic)

	Increase in skin thickness (mm)		
	48 hours post challenge	**72 hours post challenge**	**96 hours post challenge**
Periadnexal PMN cell count at 24 hours	$r_s = 0.828$ ($p<0.005$)	$r_s = 0.806$ ($p<0.005$)	$r_s = 0.838$ ($p<0.005$)

Figure 5.4: Photomicrograph of a 24 hour biopsy of a PPD lesion from a control sheep. Note the marked density of cells in the section, particularly periadnexally. These cells are primarily PMNs as shown in the inset . (H&E, x40, with inset x 250).

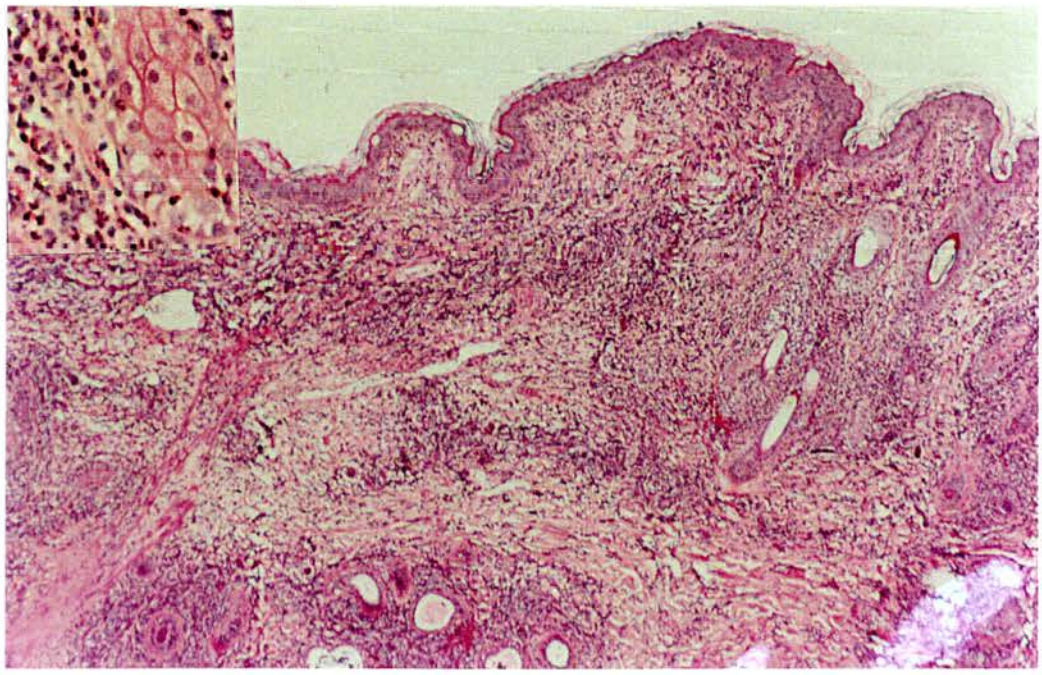
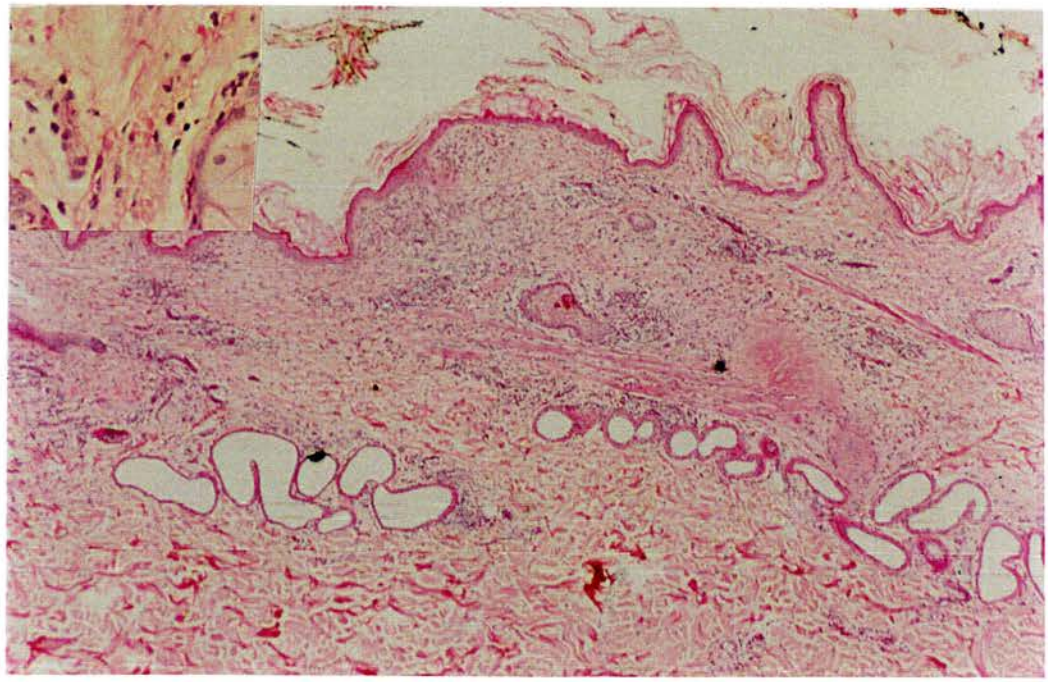


Figure 5.5: Photomicrograph of a 24 hour biopsy of a PPD lesion from an MVV infected sheep that exhibited a depressed DTH response(071). Note the much reduced density of cells in the reaction at this point, although the inset shows that these are still predominantly PMNs (H&E, x250).



5.3.4 $CD4^+$ cells

$CD4^+$ cells were found at low levels perivascularly in normal skin. In the PPD reaction, the $CD4^+$ cells were markedly increased in the 24 hour biopsy, reaching maximal levels at the 72 hour time point (Appendix 5.3.4). The levels in the MVV animals were significantly lower at the 24 hour time point, with a significance of $p<0.05$ (Figure 5.6). There were no significant differences at 72 hours post challenge, with levels of $CD4^+$ cells similar in both groups. There were significant correlations ($p<0.05$) between the periadnexal $CD4^+$ cell densities at 24 hours and the later skin thickness (Table 5.3). There were no significant alterations in the numbers of $CD4^+$ cells in the control biopsies.

Figure 5.6

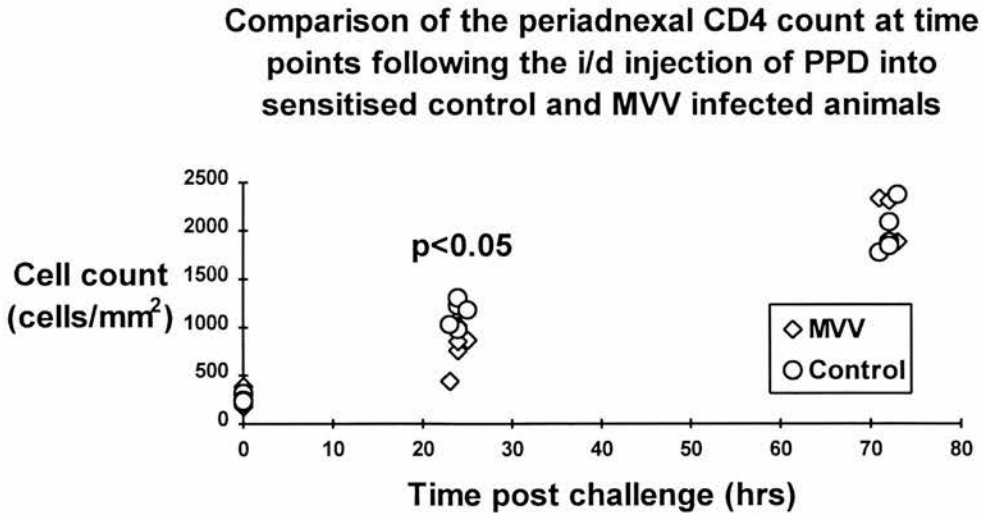


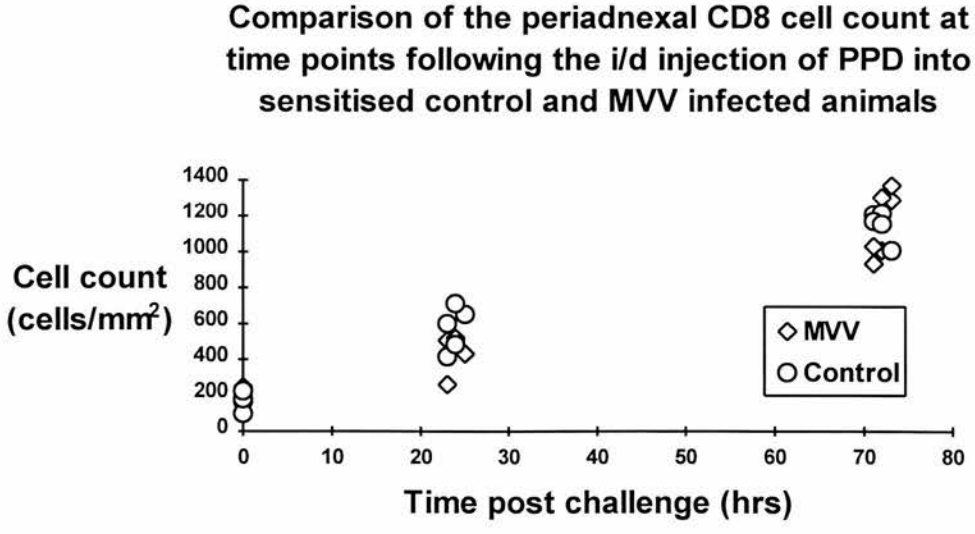
Table 5.3 Correlations between the periadnexal 24 hour cell count of $CD4^+$ cells and the increase in skin thicknesses at 48, 72 and 96 hours post challenge, incorporating the counts for both infected and control groups (Spearmann ranking correlation statistic)

	Increase in skin thickness (mm)		
	48 hours post challenge	**72 hours post challenge**	**96 hours post challenge**
Periadnexal CD4 cell count at 24 hours	$r_s = 0.642$ ($p<0.05$)	$r_s = 0.628$ ($p<0.05$)	$r_s = 0.691$ ($p<0.05$)

5.3.5 $CD8^+$ cells

In normal and control (PBS injected) biopsies, $CD8^+$ cells were distributed in a similar pattern to the $CD4^+$, being concentrated in the perivascular area, although they were present at a lower level (Appendix 5.3.5). In the PPD lesion biopsy, cell numbers were increased at 24 hours, but rose to a maximal level at 72 hours post challenge, achieving counts that were approximately half those of the $CD4^+$ cells (Figure 5.7). There was no significant difference in $CD8^+$ cell counts between the MVV and control group, and no correlation between $CD8^+$ numbers and skin thickness was present. The $CD4^+/CD8^+$ cell ratio was not significantly different between the groups of sheep

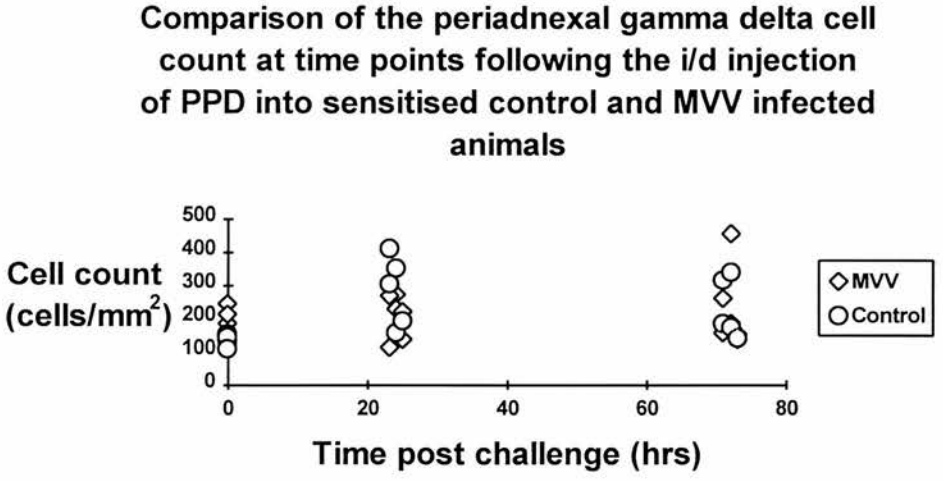
Figure 5.7



5.3.6 Gamma delta cells

Gamma/delta T cells were found at low levels, predominantly in the perivascular area, in the untreated and control (PBS injected) biopsies(Appendix 5.3.6). They remained present only at low levels in the DTH lesions, and no consistent pattern of alteration in gamma/delta cell numbers was present in these biopsies, although there was a marginal rise at 24 hours evident in some animals (Figure 5.8, Appendix 5.3.6). No significant differences between the MVV and control groups of animals, or correlations between cell count and skin thickness, were present.

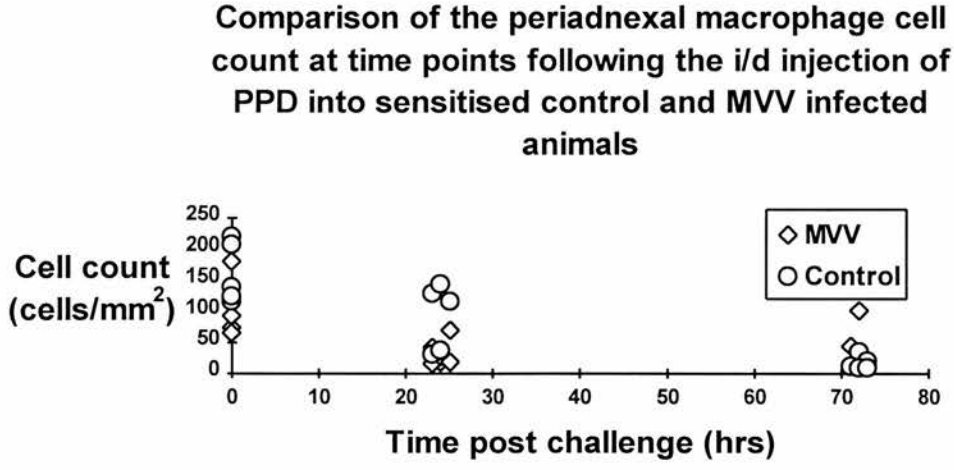
Figure 5.8



5.3.7 Macrophages

Macrophages were found at relatively low levels in normal and control (PBS injected) biopsies, with levels remaining unchanged after PBS injection in the control biopsies (Appendix 5.3.7). Macrophages constituted the smallest population of cells in the DTH lesion, with a slight rise in cell numbers at 24 hours, followed by a marked decrease reaching minimal levels at the 72 hour time point (Figure 5.9, Appendix 5.3.7). There were no significant differences in macrophage cell count between MVV and control groups, nor correlations between macrophage cell count and DTH response size present.

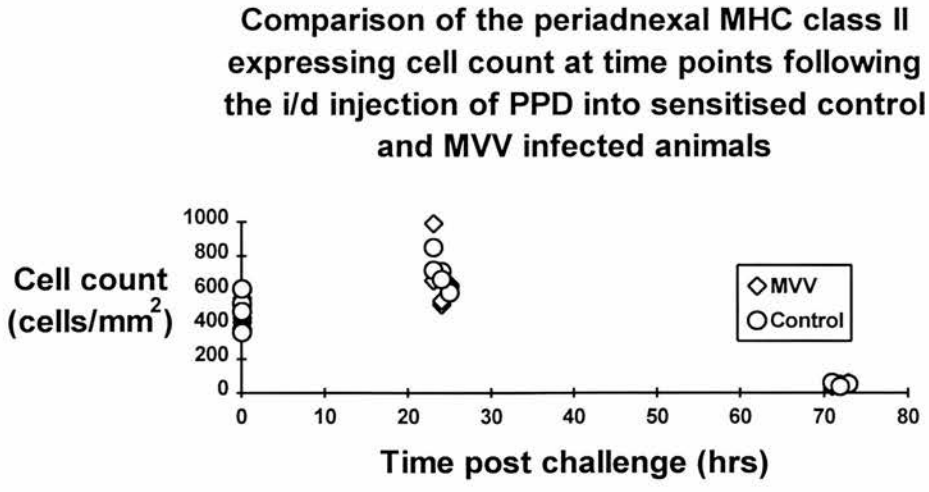
Figure 5.9



5.3.8 MHC class II expression

MHC class II expression, as defined by the number of cells staining positive for MHC class II, followed a very similar pattern to the macrophage cell staining, with cells expressing MHC class II at low levels in normal skin, and remaining unchanged in the control (PBS) injection sites (Appendix 5.3.8). In the DTH lesions, the number of MHC class II expressing cells increased slightly at the 24 hour time point, before decreasing dramatically to almost negligible levels by 72 hours (Figure 5.10, Appendix 5.3.8). There were no significant differences in the number of MHC class II expressing cells between the MVV and control groups, nor correlations between MHC class II expression and DTH skin thickness noted.

Figure 5.10



5.4 DISCUSSION

The results from this study have confirmed the findings of the previous chapter, with a significant and variable depression of the size of the DTH response in the sheep infected with MVV. This depression in the gross reaction was significantly associated with a depression in the levels of PMNs and $CD4^+$ cells in the reaction biopsies of the MVV sheep, particularly evident in the early time point biopsy of 24 hours post injection. The PMN cell count was the most significantly depressed at this point, and exhibited the strongest correlation with the size of the DTH. Counts of $CD8^+$ cells, gamma delta cells, macrophages, and MHC class II expressing cells were not statistically different between the two groups.

This depression in DTH response size has been previously recorded in sheep experimentally infected with MVV (Myer et al. 1988), although this contradicted the findings of a previous study which could find no difference in the size of a DTH reaction between experimentally infected and control animals (Larsen et al. 1982a). A depressed DTH response is a common finding in the latter stages of HIV infection, being utilised as a predictor of the progression of infection to the AIDS syndrome (Redfield et al. 1986; Colebunders et al. 1989; Borleffs et al. 1991; Johnson et al. 1992; Blatt et al. 1993; Birx et al. 1993; Pesanti, 1994), and has also been reported to occur in cats infected with FIV (Otto et al. 1993). It is surprising, therefore, that there has been no corresponding published study of the immunohistological characteristics of this depression associated either with MVV or any of the related lentiviral infections. Several studies of the response in HIV infected individuals have linked this depression in DTH with a depression in the circulating $CD4^+$ cells and associated progression of infection to the AIDS complex (Borleffs et al. 1991; Blatt et al. 1993; Birx et al. 1993). This reduction in circulating $CD4^+$ cells is not a recognised feature of MVV infection (Lujan et al. 1993), and therefore cannot provide a direct explanation for the reduced DTH. This leads to the speculation that the deficiency in

the DTH response size is related either to the mechanism of attraction of the PMN and $CD4^+$ cell into the early lesion, or the ability of these cells to enter and/or function in the lesion.

The complex nature of the PPD initiated DTH response, combined with a lack of information regarding the specific cellular interactions involved in the normal response in sheep, leads to difficulties in defining the basis of the depressed cellular infiltration into the early lesion. The fact that the reaction is already significantly different by the 24 hour time point suggests that the mechanism involved in the alteration of the response is one occurring during the initiation of the response, at a time point before there has been significant infiltration with cells.

The basis of the DTH response is the presentation of the initiating antigen, in association with MHC class II, by an antigen presenting cell to an antigen specific $CD4^+$ T helper cell. The interaction of these two cell types leads to the release of proinflammatory mediators that initiate the subsequent cellular infiltration (McKeever, 1994). There is some dispute in previous publications as to the histopathological character of the tuberculin DTH, but the present study suggests that the PMN is of crucial significance in the establishment of the gross characteristics of the DTH response, a finding that is supported by the work of Kudo et al (1993), who used monoclonal antibodies to deplete PMNs in a murine model and discovered that PMNs were essential in both the priming and initiation phase of the DTH response. The author is unaware of any work investigating the role of the PMN in the human DTH response, and the involvement of this cell type in the HIV associated DTH deficiency is unknown. The evident correlation of early PMN infiltration with the eventual maximal gross size of the DTH could be explained by the fact that they contain and produce a large range of proinflammatory mediators (Buchta, 1990), and it may be speculated that they are attracted into the lesion in a non specific manner as a result of mediators released following the initial reaction between the antigen

presenting cells and antigen specific $CD4^+$ cells, and subsequently release their mediators in situ, augmenting the inflammatory response. The decrease in PMN cells involved in the reaction at the 24 hour time point in the MVV infected sheep may be either associated with a lack of inflammatory signal, or an inability of these cells to respond to a normal signal. The fact that both the PMN and $CD4^+$ cell infiltration is decreased suggests that the deficiency is associated with a depressed or altered proinflammatory signal.

The $CD4^+$ T helper cell population is commonly accepted to be a major component of the DTH response (Poulter et al. 1982; Konttinen et al. 1983; Platt et al. 1983; Gibbs et al. 1984), with the role of release of cytokines responsible for the further mediation of the immune response (McKeever, 1994). The $CD4^+$ cell population has been studied extensively in mouse and man, where it has been found that this lymphocyte subset may be divided into two distinct groups, designated Th1 and Th2, which have been separated on the basis of their cytokine production profile. The Th1 subset is predominantly associated with DTH response, and the Th2 subset is primarily associated with the control of a humoral and/or allergic response (Mosmann, Coffman, 1989; Del Prete et al. 1991). This separation of $CD4^+$ cells into functionally distinct populations has not yet been reported in the sheep, although one study has recorded a reduction in the gross size of the tuberculin DTH response in sheep pretreated with a monoclonal antibody to interferon-γ, this cytokine being one of the main cytokines produced by Th1 type cells (Emery, Davey, 1995). Evidence from studies of the $CD4^+$ population of cells in HIV infection has revealed that, as the infection progresses to AIDS, there is a dysregulation in the function of antigen presenting cells that leads to a decrease in Th1 type cells and an increase in Th2 type cells in the circulation (Meyaard et al. 1993). This change in proportion of population of $CD4^+$ cells, as well as the overall decline in $CD4^+$ cell numbers, may explain a decrease in the ability of the individual to develop a DTH reaction. One could speculate that this mechanism may be operating in the sheep, although the existence

of the Th subtypes is not proven in this species. A shift from a predominantly Th1 type response to a Th2 type response would result in the increased production of antibody, a phenomenon which is investigated later. Lowering of Th1 cell numbers/proportions would also be linked with a decline in PMN attraction to the DTH site since interferon-γ, a Th1 associated cytokine, is one of the more important mediators of PMN infiltration(Colditz, Watson, 1992).

The macrophage is also a key component of the DTH response (McKeever, 1994), and the finding of no significant difference in macrophage cell numbers between the infected and control group does not indicate whether there are any functional alterations in this cell type as may be expected as this is the main target cell for MVV infection (Gorrell et al. 1992; Lujan et al. 1994; Brodie et al. 1995). However, only one in one million macrophages are considered to be infected (Brownlee, personal communication). There are various studies relating macrophage association with HIV or HIV peptides with altered cytokine production *in vitro*, of which the constitutive overexpression of IL-10 by infected macrophages (Akridge et al. 1994; Emilie et al. 1994; Haraguchi et al. 1995) would provide a plausible solution to the puzzle of the declining DTH, since IL-10 is both involved in the switch between Th1 and Th2 subsets and has been shown to inhibit DTH reactions (Powric et al. 1993; Li et al. 1994). The DTH defect, however, may be associated with the altered production of a large range of inflammatory mediators by the macrophage, as they are responseible for the production of many such products.

The lack of significant difference in the $CD8^+$ cell counts, and gamma delta cell counts correlates with the lower number of these cells found in the lesion, and suggests that they have a lesser part to play in the release of mediators responsible for the development of the gross DTH lesion, although absolute numbers once again do not give an indication of possible functional differences.

In conclusion, these experiments have indicated that the depression in the gross size of the DTH response in sheep infected with MVV is significantly associated with the level of PMN and $CD4^+$ cells present in the early DTH lesion. This leads to speculation that there is either a deficit in the mechanism of attraction of these cells into the lesion, or a deficiency of migration into and/or function of these cells in the lesion.

CHAPTER SIX

THE EFFECT OF SELECTIVE PMN DEPLETION ON THE DTH REACTION

6.1 INTRODUCTION

Previous chapters have described the finding of depression in the gross size of a DTH response in sheep seropositive to MVV, and significantly associated this alteration in gross characteristics with a decreased density of PMNs and $CD4^+$ cells in the reaction at an early time point. The presence of considerable numbers of PMNs in the early DTH reaction has never been previously reported in sheep, although there have been variable reports of PMNs in the early DTH reaction of other species. The association of PMNs with the failure of development of the gross characteristics of a DTH response has been previously investigated in the rat, using monoclonal antibodies to deplete circulating PMNs at both the priming and initiation phase of a DTH model (Kudo et al. 1993a; Kudo et al. 1993b). This work indicated that the PMN was a crucial cell in the establishment of a grossly visible DTH response, although the DTH model used in these experiments was markedly different from the classical tuberculin response in sheep used by the author in the previous experiments.

In order to confirm that the PMN was indeed an integral component of the classical tuberculin DTH reaction in sheep, and to correlate the findings of lowered levels of PMNs in the early reaction being associated with the reduction in size of the DTH response seen in MVV seropositive animals, it was important to establish a direct link between the PMN cell and the development of a typical tuberculin response. The most direct method of investigating this link was the specific depletion of circulating PMNs immediately prior to the induction of a standard DTH response. The most specific method of achieving depletion would be the use of a PMN specific

monoclonal antibody which has been used in other species, but unfortunately there is no such ovine reagent available. Previous authors had reported achieving a PMN depletion in sheep through the use of the cytotoxic drug hydroxyurea, which had been shown to mainly reduce the number of circulating PMNs, with a relatively minor depletion of the level of circulating lymphocytes (Heflin, Brigham, 1981; Raj et al. 1985; Kubo et al. 1992; Pearse, Sylvester, 1992).

The following experiment was therefore undertaken utilising hydroxyurea to deplete circulating PMNs in a group of control sheep, previously primed with BCG vaccine, at the time of initiation of the DTH response with a PPD challenge. In order to confirm the specificity of the hydroxyurea induced changes, the level of circulating blood cells was closely monitored during the depletion and subsequent DTH using routine haematology and FACScan flow cytometry techniques.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Six adult female Texel sheep (Table 6.1) were obtained from a flock that was a participant in the UK sheep health scheme, and had therefore been seronegative for MVV for a number of years. The sheep were seronegative for MVV when tested immediately prior to the commencement of the experiment.

Table 6.1 Details of the sheep used in this experiment

Sheep Number	Breed	Age (years)	Sex
RT 33	Texel	7	Female
RT 34	Texel	5	Female
RT 35	Texel	6	Female
RT 36	Texel	7	Female
RT 37	Texel	5	Female
RT 38	Texel	5	Female

The control sheep detailed in chapter 5 (sheep 183, 184, 245, 246, 247) were used as a control group in this experiment.

6.2.2 DTH initiation, measurement and biopsy

The DTH was initiated, measured, and biopsied in the standard manner described in 2.4 - 2.6.

6.2.3 Immunohistopathological staining and quantification

Routine paraffin embedded sections were processed and stained with haematoxylin and eosin and toluidine blue as described in chapter 2.

Frozen sections were processed routinely and stained with monoclonal antibodies SBU-T4 (CD4), SBU-T8 (CD8), 86D ($\gamma\delta$ T cell), SW73.2 (pan MHC class II) and OM-1 (CD11c, macrophage) (chapter 2).

Quantification of cellular infiltration was performed utilising the computerised image analysis system previously described in 5.2.5.

6.2.4 FACS analysis of peripheral lymphocytes

Peripheral blood was collected from the sheep for FACS analysis at the commencement of the PMN depletion therapy, and immediately prior to the initiation of the DTH with PPD as described in 2.9.1. The cells were stained with monoclonal antibodies SBU-T4 (CD4) and SBU-T8 (CD8), as well as normal mouse serum and an irrelevant monoclonal antibody of identical isotype (VPM53, an anti-campylobacter like organism monoclonal antibody (McOrist et al. 1987)) in the manner described in 2.9.2. Cells were analysed using a FACScan automated cell counter as described in 2.9.3.

6.2.5 PMN depletion

Circulating PMNs were selectively depleted from the circulation in the manner previously described by other workers. Hydroxyurea (Hydrea capsules, Bristol-Myers Squibb Pharmaceuticals Ltd., Dublin, Ireland) was dissolved in a 0.9% w/v sterile saline solution (Aquapharm, Animalcare Ltd., York, UK) at a rate of 4g of hydroxyurea to 60 ml of saline solution. A 60 ml (4g) aliquot of the solution was then administered to the sheep intravenously daily for a period of six days, commencing six days prior to the challenge of the sheep with PPD, such that maximal depletion of PMNs had occurred at the time of challenge.

6.2.6 Routine haematological monitoring

The level of circulating blood cells was monitored daily throughout the depletion period and subsequent DTH response, with peripheral venous blood collected and analysed in a standard manner (2.3).

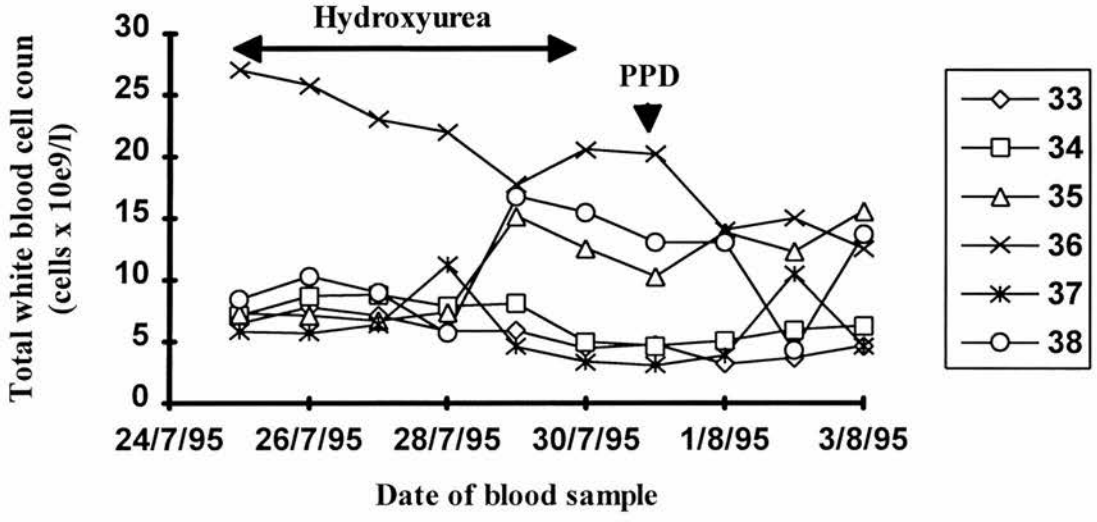
6.3 RESULTS

6.3.1 Haematological changes following hydroxyurea treatment

Considering firstly the total white blood cell counts, at the start of the depletion experiment sheep 35 and 36 had cell counts above the laboratory normal range with the others having counts within the normal range. In response to hydroxyurea treatment, there was initially a steady fall in the number of white blood cells in all the sheep, although sheep 36 had a count that was very high and remained so throughout the experiment. After four days of treatment, sheep 35 and 38 exhibited a rapid increase in the cell count, and subsequently remained at levels above normal for the rest of the experiment. The cell counts of sheep 33, 34 and 37 remained steadily declining throughout the treatment, although sheep 37 was the only sheep to have a subnormal cell count at the time of PPD injection (Figure 6.1 and Appendix 6.3.1).

Figure 6.1

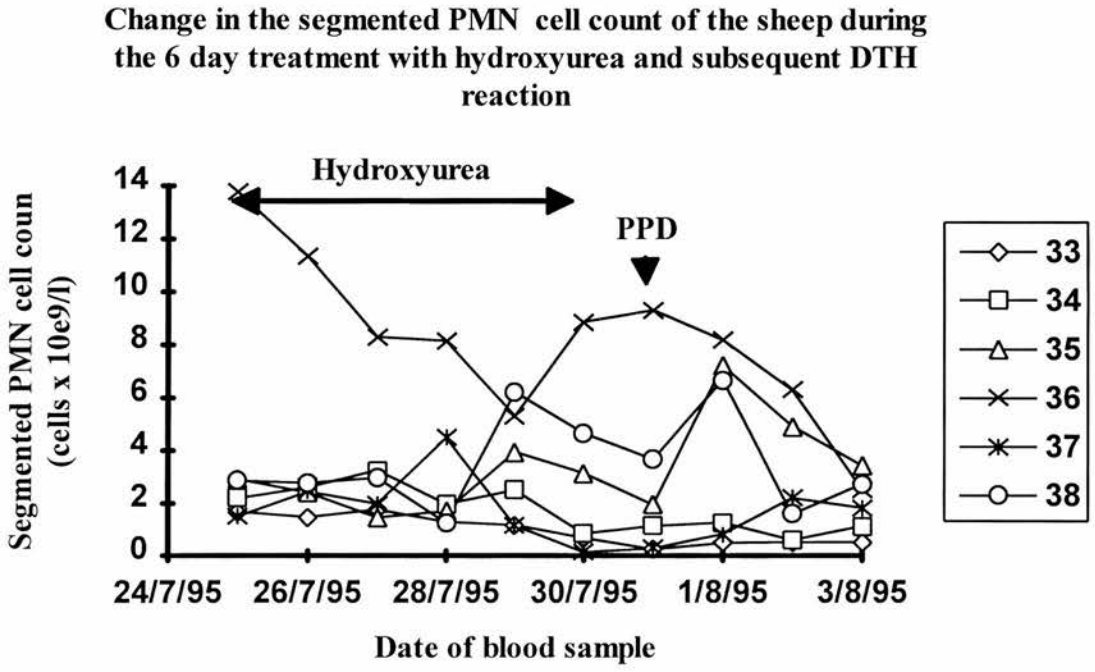
Change in the total white blood cell count of the sheep during the 6 day treatment with hydroxyurea and subsequent DTH reaction



Although there was a trend towards a lower total white blood cell count in the depleted group, there was no significant difference between the treated and control groups at the time of initiation of the DTH, or correlation between the cell count and increase in skin thickness.

The segmented PMN cell count followed similar trends to the total white blood cell count. Sheep 36 had a very high cell count at the commencement of hydroxyurea treatment which gradually declined, but with a marked increase after 5 days of treatment and remaining above normal levels throughout the experiment. Sheep 35 and 38 once again showed a gradual decline in cell numbers for the first four days of hydroxyurea treatment, but then exhibited a sudden rise in cell numbers, which then fell slightly once more at the time of DTH initiation, but then rose once more during the early stages of the DTH. Sheep 33, 34 and 37 exhibited a gradual decline in PMN numbers, and the counts of sheep 33 and 37 were subnormal at the time of DTH initiation (Figure 6.2 and Appendix 6.3.1).

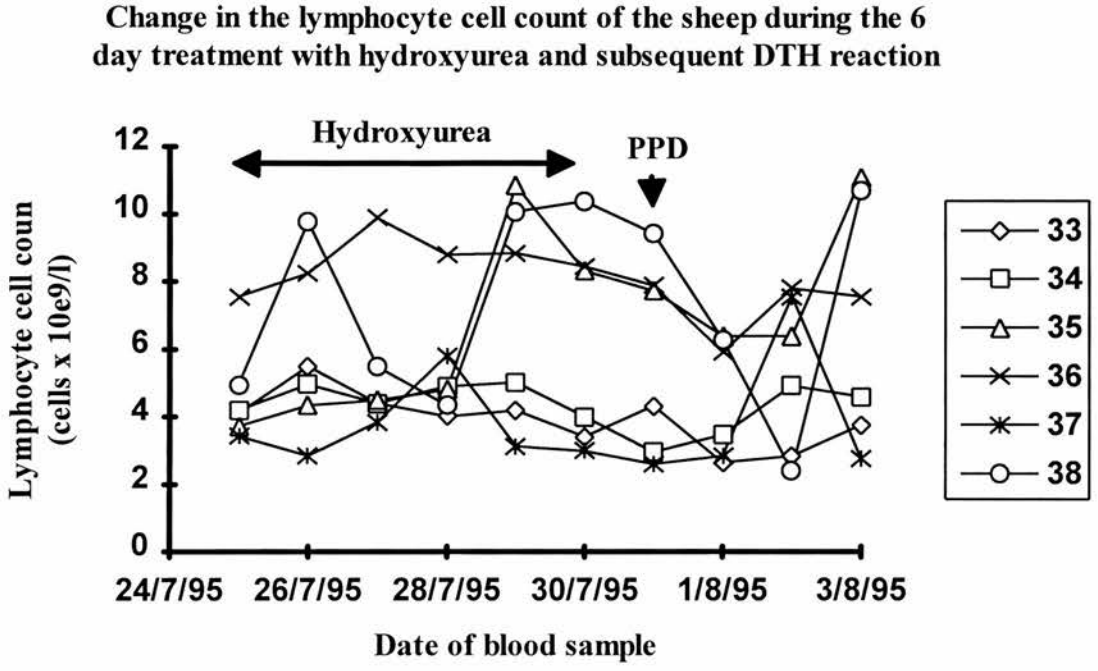
Figure 6.2



There was no significant difference between the PMN cell counts in the depleted group and the control group at the time of initiation of the DTH, or correlation between the cell counts and increases in skin thickness.

The lymphocyte cell counts appeared to be relatively unaffected by the hydroxyurea, but once again sheep 36 had levels above normal throughout the experiment. Sheep 35 and 38 exhibited also exhibited a sharp rise in lymphocyte numbers after 4 days which then declined during the DTH response (Figure 6.3 and Appendix 6.3.1).

Figure 6.3



Once again, there was no significant difference in the lymphocyte cell counts between the depleted and control groups, with no correlations between the cell counts and increases in skin thickness.

The eosinophil cell counts of all sheep except 36 were relatively low and appeared to be unaffected by hydroxyurea treatment. Sheep 36 had a very high eosinophil cell count that, although declining, remained above normal levels throughout the majority of the experiment (Appendix 6.3.1). There was no significant difference in the eosinophil cell counts between the depleted and control groups, with no correlations between the cell counts and increases in skin thickness.

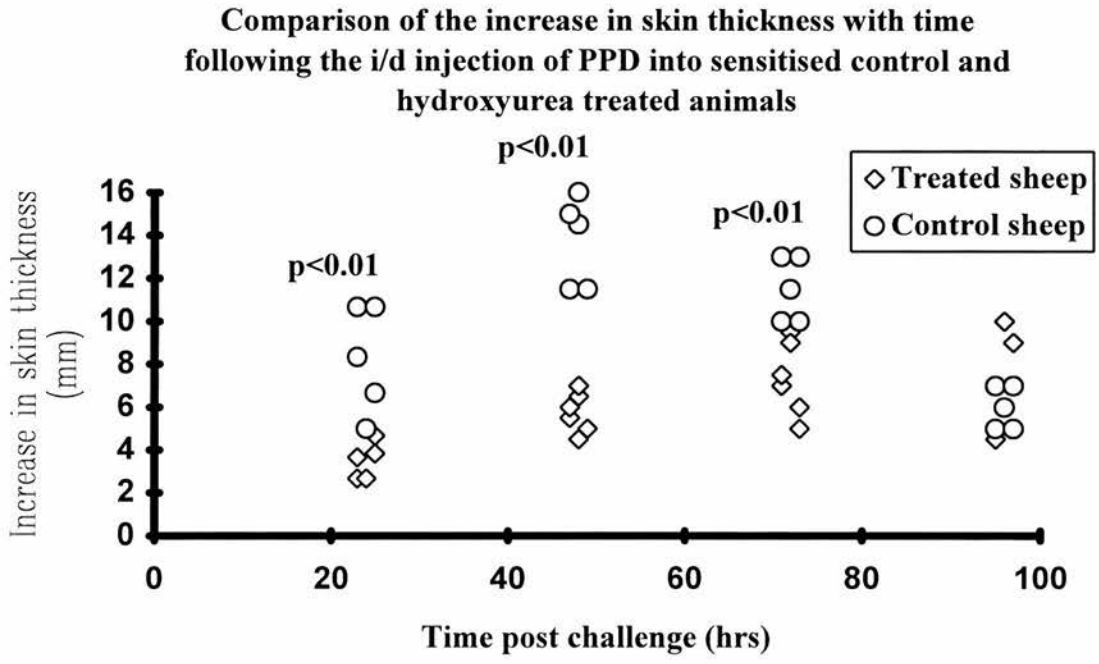
Monocyte counts were also relatively unaffected by the hydroxyurea treatment (Appendix 6.3.1).

The FACScan data produced figures for the relative proportion of lymphocytes that were $CD4^+$ or $CD8^+$ at the start of hydroxyurea treatment and at the initiation of the DTH. There was a general increase in the level of $CD4^+$ cells and a general decrease in the levels of $CD8^+$ cells following the hydroxyurea treatment, although this was found to be not significant using a Wilcoxon statistic. The ratio of $CD4^+/CD8^+$ lymphocytes was significantly increased ($p<0.05$, Wilcoxon statistic) after the hydroxyurea treatment (Appendix 6.3.1). No FACScan data was available for the control group.

6.3.2 Increase in skin thickness of the DTH lesion

The increases in skin thicknesses were variable within the group, with the increases in skin thicknesses for the treated group being significantly lower than the control group from 24 to 72 hours post challenge (Figure 6.4 and Appendix 6.3.2).

Figure 6.4



6.3.3 Histopathological characterisation of the DTH lesion

Qualitatively, the DTH reaction was similar in the PMN depleted sheep to the previously described normal DTH reaction, although there was an apparent decrease in the density of PMNs in the early DTH reaction in this treated group.

The PBS injected control skin biopsies were once again unremarkable, with no apparent alteration from the skin biopsies taken from non-injected sites.

Figure 6.5: Photomicrograph of a 24 hour biopsy taken from the PPD lesion of a control sheep. Note the marked accumulation of infiltrating cells in the periadnexal region (H&E, x40).

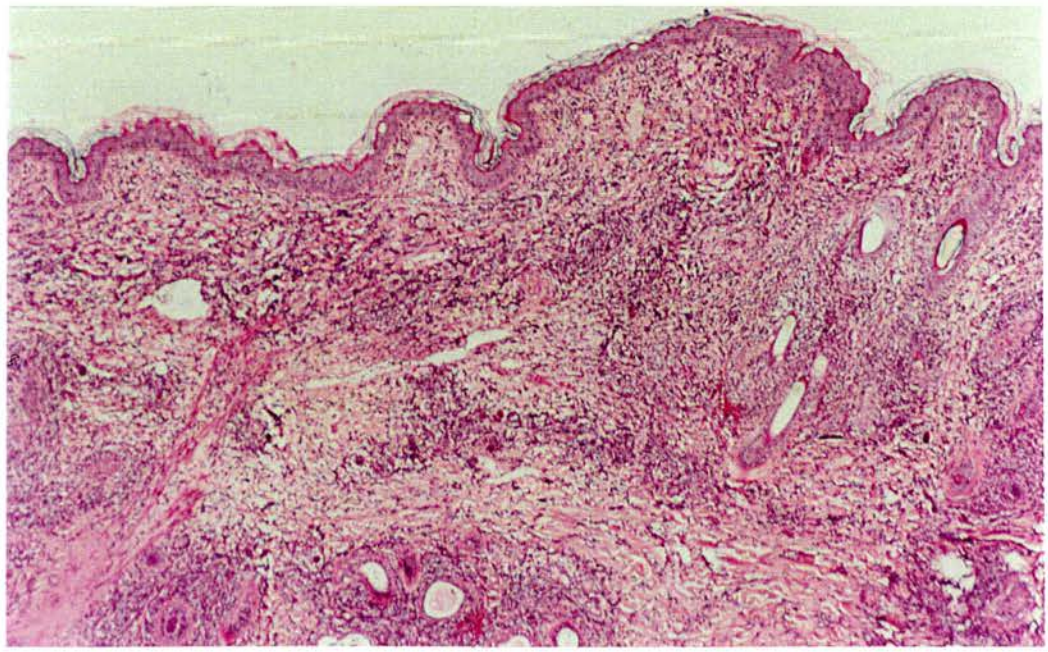
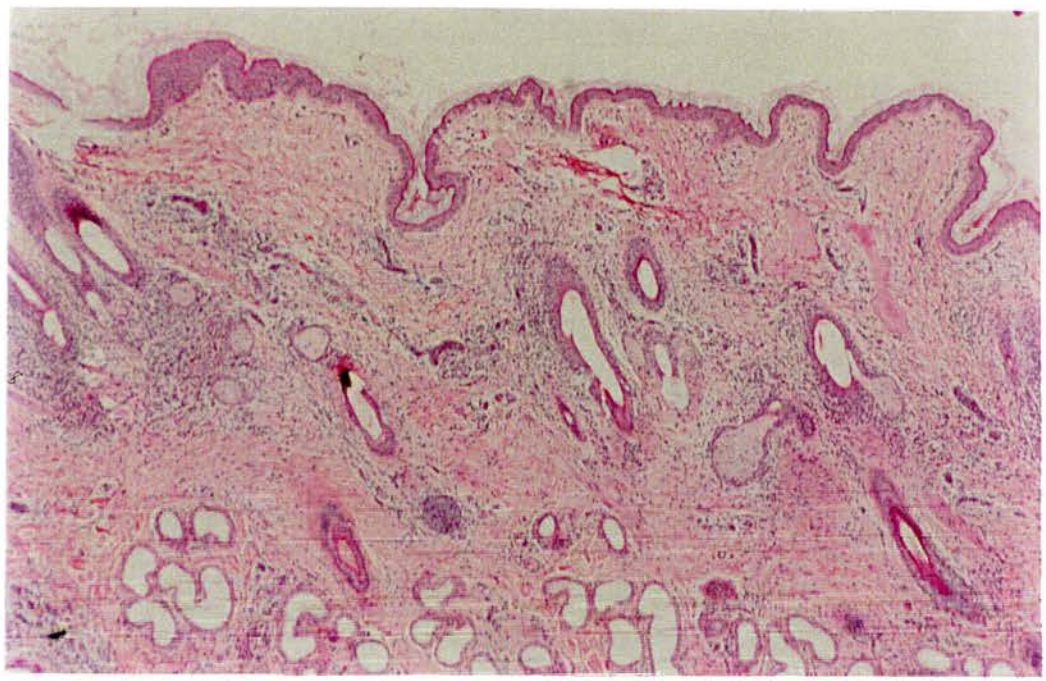


Figure 6.6: Photomicrograph of a 24 hour biopsy taken from the PPD lesion of a PMN depleted sheep (34). Note the marked reduction in the density of infiltrating cells. The reduction is associated with a lower count of PMNs in the lesion (H&E, x40).



6.3.4 PMN cell count

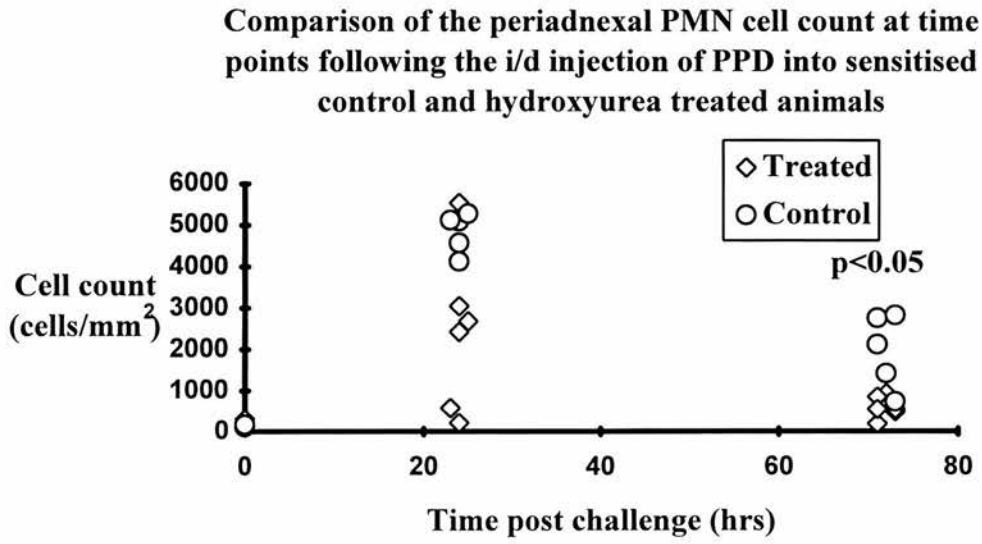
Considering the DTH reaction, PMNs were present in the biopsies at low levels initially, rose to a maximal level at 24 hours at which point they were the predominant cell, before the numbers fell again at 72 hours. The sheep in the hydroxyurea treated (PMN depletion) group exhibited markedly variable PMN cell counts, which were usually below the more consistent PMN cell count in the control group, although this reduction in PMN cell numbers was only significant in the periadnexal compartment at 72 hours (Figure 6.5, 6.6, and 6.7 and Appendix 6.3.4). When the data for both groups was combined, there were significant correlations between the PMN cell counts and increases in skin thicknesses (Table 6.1). There were also significant correlations ($p<0.05$, Spearman statistic) between the circulating PMN cell count and the density of PMNs in the superficial dermis of the DTH reaction at both 24 and 72 hours.

Table 6.1 indicating the significant correlations between PMN cell counts and increases in skin thicknesses with data from both the PMN depleted and control groups combined (Spearmann statistic).

	Increase in skin thickness (mm)		
	48 hours post challenge	**72 hours post challenge**	**96 hours post challenge**
Periadnexal PMN cell count at 24 hours	$r_s = 0.729$ $p<0.02$	$r_s = 0.653$ $p<0.05$	NS
Dermal PMN cell count at 24 hours	$r_s = 0.679$ $p<0.05$	$r_s = 0.621$ $p<0.05$	NS
Periadnexal PMN cell count at 72 hours	ND	$r_s = 0.694$ $p<0.05$	NS

The PBS injected control biopsies exhibited little alteration from the non injected skin, although there was a significantly higher PMN cell count in the 24 hour periadnexal biopsies taken from the PMN depleted group (Appendix 6.3.4).

Figure 6.7

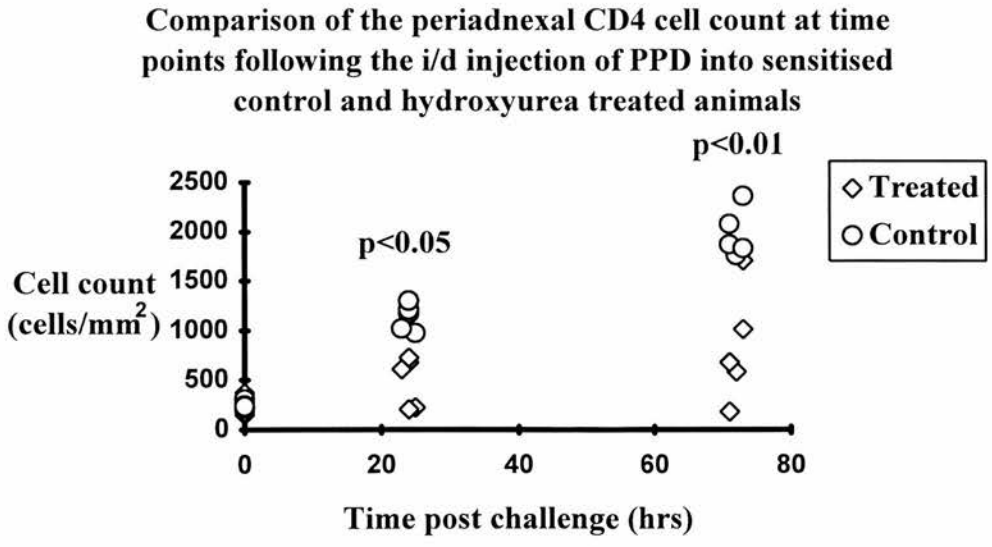

Comparison of the periadnexal PMN cell count at time points following the i/d injection of PPD into sensitised control and hydroxyurea treated animals
Treated
Control
p<0.05
Cell count (cells/mm2)
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
Time post challenge (hrs)

6.3.5 $CD4^+$ cell count

Considering the DTH reaction, the $CD4^+$ cell counts were at low levels in the control skin biopsies of both groups, rising at 24 hours, and becoming maximal at 72 hours. The $CD4^+$ cell counts were significantly depressed in the animals that had been hydroxyurea treated at both 24 and 72 hours (Figure 6.8 and Appendix 6.3.5). When the data for both groups was combined, there was a significant correlation between the periadnexal $CD4^+$ cell counts at 24 hours and the increase in skin thicknesses at 24 hours ($p<0.05$, Spearmann statistic).

The $CD4^+$ cell counts in the PBS injected sites remained at low levels with no significant differences between the groups (Appendix 6.3.5).

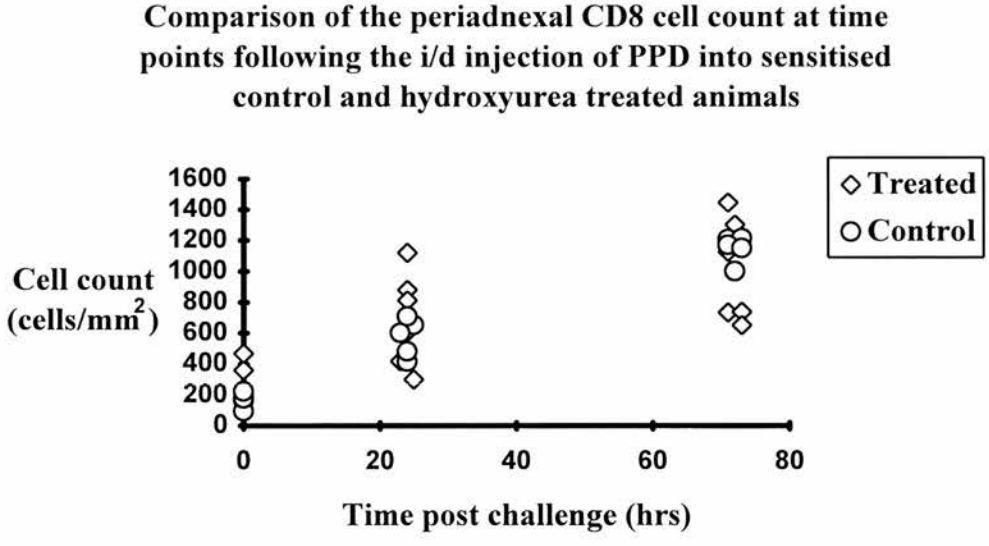
Figure 6.8



6.3.6 $CD8^+$ cell count

The counts of $CD8^+$ cells in the DTH reaction were very similar between the two groups, with the cell numbers increasing at 24 hours and becoming maximal at 72 hours (Figure 6.9 and Appendix 6.3.6). Cell numbers remained relatively unchanged in the PBS injected sites (Appendix 6.3.6). There were no significant differences in counts in the DTH reaction or PBS injected sites. There were no correlations between the $CD8^+$ cell counts and the increases in skin thicknesses in the DTH lesion.

Figure 6.9

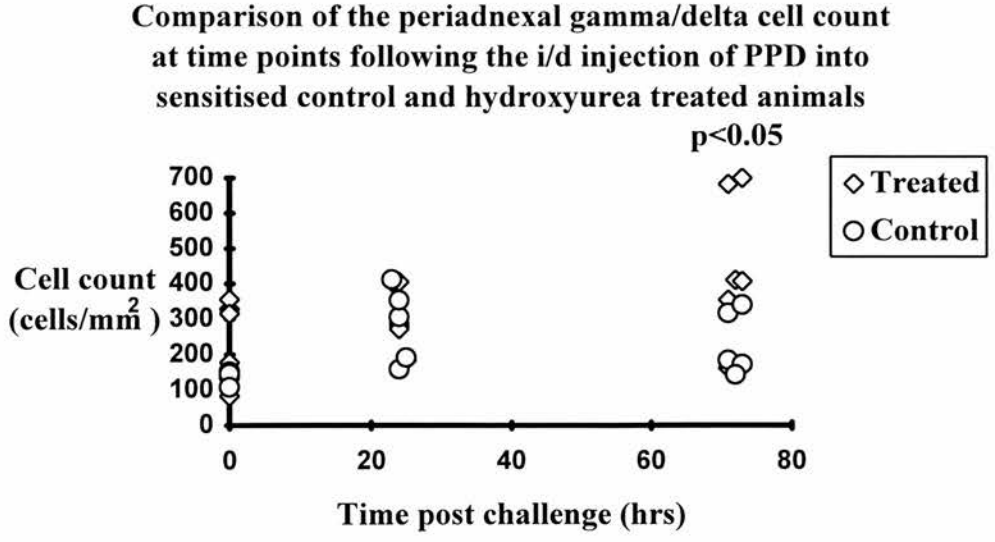


6.3.7 Gamma-delta cell count

Considering the DTH reaction, the counts of gamma/delta cells were initially very similar between the groups, although by 72 hours the cell count in the depleted group had risen to be significantly higher than the control group (Figure 6.10 and Appendix 6.3.7). When the data for both groups was combined, there was a significant negative correlation between the periadnexal gamma/delta cell counts at 72 hours and increases in skin thickness at 72 hours ($p<0.05$, Spearmann statistic).

Within the PBS injected sites, there was relatively little change in cell numbers, although there were significantly higher gamma/delta cell counts at 24 hours in the depleted group.

Figure 6.10



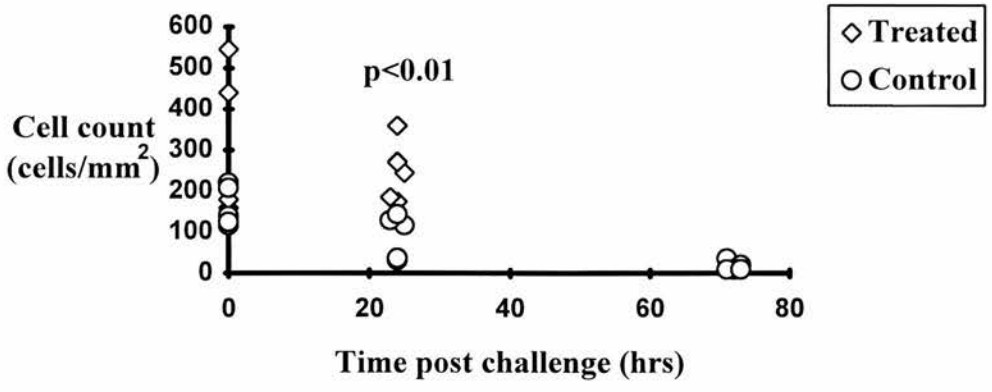
6.3.8 OM1 positive cell count

Considering the DTH reaction, the levels of OM1 positive cells was relatively low in both groups in the non injected skin and at 24 hours, falling to almost negligible levels by 72 hours. The depleted group had significantly higher counts ($p<0.05$, Mann-Whitney U statistic) of OM1 positive cells at 24 hours (Figure 6.11 and Appendix 6.3.8).

There was relatively little change in OM1 cell counts in the PBS injected sites, with no significant differences present.

Figure 6.11

Comparison of the periadnexal OM1 cell count at time points following the i/d injection of PPD into sensitised control and hydroxyurea treated animals

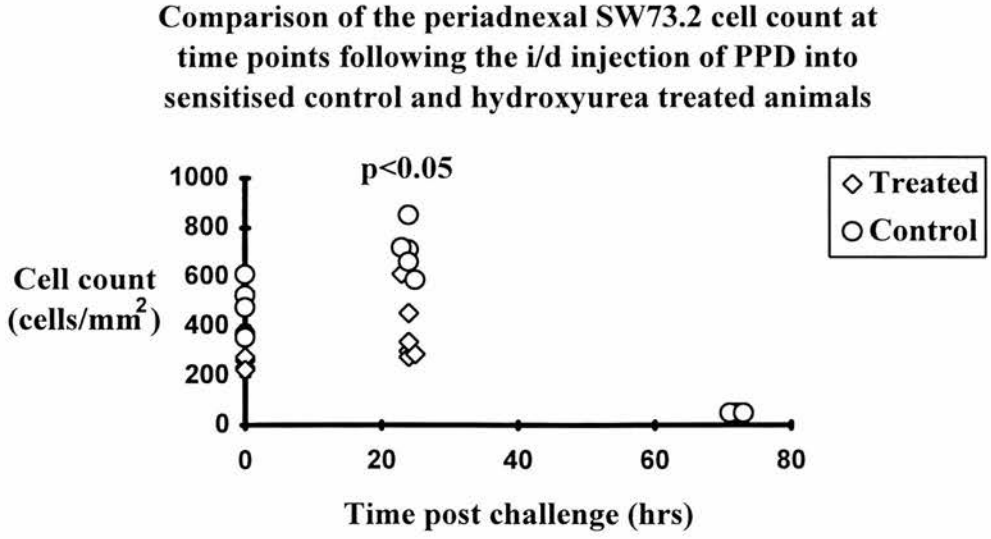


6.3.9 MHC class II expressing cell count

Considering the DTH reaction, there were relatively low levels of SW73.2 expressing cells in the early biopsies, falling to very low levels at 72 hours. The depleted group had significantly lower counts ($p<0.05$, Mann-Whitney U statistic) of SW73.2 positive cells at 24 hours (Figure 6.12 and Appendix 6.3.9). There were no significant correlations between the SW73.2 cell counts and increases in skin thickness.

There was relatively little change in SW73.2 cell counts in the PBS injected sites, with no significant differences present.

Figure 6.12



6.4 DISCUSSION

The results of this experiment suggest that the use of hydroxyurea to selectively deplete the level of circulating PMNs in sheep is not as reliable as previously reported (Heflin, Brigham, 1981; Raj et al. 1985; Kubo et al. 1992; Pearse, Sylvester, 1992), with three sheep exhibiting a marked increase in the circulating level of both white blood cells and PMNs after 4 days (sheep 35 and 38) and 5 days (sheep 36) respectively. This is indicative of the development of an acute inflammatory response, and would suggest that the depletion of PMNs in the early part of the experiment has allowed the evolution of a subclinical infective process. Although the precise nature of this infection was not evident on clinical inspection, several of the sheep had undergone recent treatment for infections in the feet and it is possible that there had been a resurgence of this infection following PMN depletion. This finding also indicates that there are mechanisms present in the sheep that are capable of overcoming the cytotoxic effects of hydroxyurea and causing an increase in circulating PMNs. Ideally, gnotobiotic animals could have been employed to reduce these problems of subclinical infection, but such animals could not have been considered true controls for the MVV seropositive animals in terms of their previous management and possible previous exposure to mycobacterial and other pathogens. The other three sheep, however, did exhibit a fall in circulating PMN numbers in response to the hydroxyurea, with one sheep becoming subnormal at the time of DTH initiation.

The hydroxyurea did appear to be somewhat selective in its depletion of peripheral PMNs, with the level of circulating lymphocytes, eosinophils and monocytes remaining relatively static during the treatment. The levels of $CD4^+$ and $CD8^+$ cells were statistically unchanged by the treatment, although the trend towards higher levels of $CD4^+$ and lower levels of $CD8^+$ cells was manifest as a significant increase in the $CD4^+/CD8^+$ cell ratio.

The failure of the hydroxyurea to deplete the level of circulating PMNs in all the experimental sheep led to a situation where there was no significant difference in the PMN cell count between the depleted and control groups, although the statistics were particularly skewed by the very high cell count of sheep 36. When the statistic was repeated excluding the three sheep that had experienced a rise in circulating PMN cells, again there was no significant difference. This reflects the low numbers of sheep in the groups, with significance difficult to attain even with marked differences between the groups.

There was, however, a significant depression in the later increases in skin thickness in the treated group, with a trend towards lower increases in skin thicknesses being associated with lower levels of circulating PMNs, although there was no statistically significant correlation present between these two parameters. This depression in the later increase in skin thickness was significantly associated with a depression in the density of PMNs in the early lesion, a result identical to that found in the depressed DTH response of the MVV seropositive sheep. The lower density of PMNs in the DTH lesion was also significantly associated with the level of circulating PMNs. These latter two significant associations indicate a circuitous relationship between the circulating PMN cell count and the increase in skin thickness in the sheep, providing further evidence for a link between the circulating PMN and the DTH reaction. The finding of a link between the circulating PMN and the DTH reaction size is concordant with the previous experiments in the rat DTH model (Kudo et al. 1993a; Kudo et al. 1993b), and indicates that the early migration of PMNs into a DTH lesion is crucially involved in the development of the later cell influx and also the development of the gross swelling associated with this reaction. Since the PMN is considered to operate in a non antigen-specific manner, this finding suggests that an earlier antigen specific interaction is involved in the attraction of PMNs to the site of antigen deposition, these PMNs then being involved in the non antigen specific augmentation of the reaction. The association with the density of the early PMN cell

infiltrate with the gross size of the DTH response reflects the wide range of proinflammatory mediators released by these cells (Buchta, 1990), particularly those associated with the control of vascular permeability (Wedmore, Williams, 1981; Abe et al. 1990; Yi, Ulich, 1992).

The depleted sheep also exhibited a depression in the density of $CD4^+$ cells in the reaction, both at 24 and 72 hours, which occurred even though treatment caused no decrease in the circulating lymphocyte count and a relative increase in the percentage of $CD4^+$ lymphocytes. There was no significant association between the reaction $CD4^+$ cell density and the circulating levels of either lymphocytes or PMNs, but the hydroxyurea treatment produces it's main effect on the level of circulating PMNs, and this depression in $CD4^+$ cell density in the DTH reaction is apparently linked to alterations in the PMN cell count. The decreased density of $CD4^+$ cells in the DTH lesion is analogous to the situation found in the MVV seropositive sheep, where a similar phenomenon occurred. The previous work investigating the association between the level of circulating PMNs and the characteristics of a DTH type response (Kudo et al. 1993a; Kudo et al. 1993b) did not use immunohistological techniques to identify particular cell types, but grouped cells morphologically as mononuclear type cells. This work did, however, indicate a depression in the mononuclear cell infiltration into DTH sites following depletion of circulating PMNs. The authors considered that this depression was linked to the fact that PMNs in the lesion are capable of releasing cytokines that are potent attractants for lymphocytes, citing IFNα as a prime candidate in this role. This hypothesis that PMNs in the lesion are responsible for the release of mediators and subsequent attraction of mononuclear cells is also suggested by the results of this experiment, with a circulating PMN suppression being linked with a decline in $CD4^+$ cells in the lesion. The specific nature of the mediators released by the PMNs and involved in this $CD4^+$ attraction are not immediately obvious, with a wide range of putative candidates being available.

The level of circulating $CD8^+$ cells appeared to be relatively lowered following the treatment with hydroxyurea when compared to the $CD4^+$ levels. There was, however, no difference between the density of these cells in the DTH lesion between the depleted and control groups. Again, this is a situation analogous with the depression in DTH response found in the MVV seropositive group. The density of gamma delta cells in the DTH reaction was slightly, but significantly, increased in the treated group. This increase was not seen in the MVV seropositive group, although the relatively low level of gamma-delta cell involvement was a common feature. The apparent lack of alteration in the level of infiltration of $CD8^+$ lymphocytes and increase in gamma-delta cell infiltration into the DTH following PMN depletion suggests that the mediators released by the PMN that are responsible for the control of $CD4^+$ cell infiltration are specific for this cell type. It also supports a hypothesis that the $CD8^+$ and gamma-delta T cells are not involved in the development of the gross characteristics of the DTH response.

The data regarding the OM1 positive cells and the SW73.2 positive cells is a little confusing, as there is an apparent reciprocal relationship between the cell densities in the control and treated groups. It would be expected that the two monoclonal antibodies would be defining the same cell populations, namely macrophage type antigen presenting cells (APCs), as the only non APC cell population that expresses MHC class II is the B lymphocyte subset which was not found in the DTH response. The actual numerical values of the cell counts, as opposed to the non-parametric rank, are very similar in the control and depleted group suggesting that the statistical difference is possibly a quirk of the results.

In conclusion, the treatment of sheep with hydroxyurea did not produce a consistent reduction in the circulating levels of PMNs. It did, however, produce evidence that the circulating level of PMNs is associated with the size of a DTH response and the level of PMNs and $CD4^+$ cell infiltration into the histological DTH

reaction. In MVV infected sheep, however, the depression in DTH response was not associated with lowered levels of circulating PMNs. This depression in DTH size following hydroxyurea treatment was not associated with a reduced influx of $CD8^+$ or gamma delta T cells into the lesion. The reduction in DTH response achieved by the treatment with hydroxyurea was remarkably similar to the reduction in DTH response associated with MVV seropositivity.

CHAPTER SEVEN

ASSESSMENT OF *IN VIVO* PMN AND $CD4^+$ CELL FUNCTION

7.1 INTRODUCTION

The previous chapters have indicated that PMNs are present in high numbers in the early DTH reaction, and that the depletion of neutrophils *in vivo* is associated with a diminished ability of the sheep to establish a DTH response. The association of PMNs with the establishment of a DTH response reflects the involvement of this cell type in the control of increased vascular permeability (Wedmore, Williams, 1981; Abe et al. 1990; Yi, Ulich, 1992), and the ability of the PMN to release a broad range of proinflammatory mediators (Buchta, 1990). The $CD4^+$ T cell was also found to be present at significant levels in the early DTH lesion, this cell being both a direct source of proinflammatory mediators, and responsible for the stimulation of the release of such mediators by other cells (Scott, Kaufmann, 1991). This corresponds with the finding that the depressed DTH response in animals chronically infected with MVV is associated with a decreased level of migration of PMNs and $CD4^+$ cells into the early reaction. This reduced migration could be associated with either a decline in the level of proinflammatory signal attracting the PMNs and $CD4^+$ cells into the lesion, or a decline in the ability of these cells to respond to a normal level of signal.

There has been no previously published investigation into the function of PMNs in sheep infected with MVV, although studies of HIV infected individuals have found reductions in the in vitro performance of PMNs particularly in patients progressing rapidly to AIDS (Gabrilovich et al. 1994), and dysregulated L selectin shedding after cytokine stimulation by PMNs in AIDS patients (Elbim et al. 1994). A study investigating the PMN function in HIV infected chimpanzees also discovered a

decline in *in vitro* PMN function in these animals (Lafrado et al. 1989). There are also reports of lentivirally associated neutropenias, particularly marked in FIV infection (Mandell et al. 1992; Linenberger et al. 1995).

Similarly, there are no published reports of $CD4^+$ cell trafficking dysfunction in MVV infected sheep, although they are present at relatively low levels in the predominant pathological lesions in the lung tissue (Cordier et al. 1992; Lujan et al. 1993).

It was important, therefore, to assess the migratory ability of PMNs and $CD4^+$ cells in the MVV infected sheep in order to ascertain whether the decrease in PMN and $CD4^+$ cell numbers in the early DTH in these animals was associated with a decreased ability of these cells to migrate into the lesion. Previous publications had shown that the dermal injection of a range of mediators were capable of recruiting PMNs and/or $CD4^+$ cells to the site of injection in sheep (Colditz, Movat, 1984; Colditz, Watson, 1992; Mulder, Colditz, 1993; Colditz et al. 1994; Seow et al. 1994). Three of these were selected for use in this trial, based on the results of these previous publications that had shown these particular mediators to predominantly attract PMNs, but also to attract smaller numbers of $CD4^+$ cells. These mediators should, therefore, mimic the cell traffic present in the early DTH lesion, where the MVV associated abnormality in cell traffic was occurring. Three distinct mediators were used to provide comparative data, as the mode of action of these three mediators has been shown to be different.

The first of these mediators was zymosan activated plasma (ZAP), which is prepared from sheep plasma, and contains activated complement components. This agent acts as a direct chemoattractant, not requiring an intermediate protein synthesis stage, and acts as an extremely potent chemoattractant of PMNs, but also attracts a

small number of $CD4^+$ cells (Colditz, Movat, 1984; Colditz, Watson, 1992; Mulder, Colditz, 1993; Colditz et al. 1994).

The second mediator used was recombinant human IL-8 (rh-IL8). This again has been shown to be a potent chemoattractant for PMNs and $CD4^+$ cells *in vivo* (Colditz, Watson, 1992; Mulder, Colditz, 1993; Colditz et al. 1994; Seow et al. 1994), with a similar potency to recombinant ovine IL-8 (roIL8) (Seow et al. 1994). Again the mechanism of attraction appears to be independent of de novo protein synthesis.

The final mediator chosen was tumour necrosis factor α (TNFα), which again is a powerful chemoattractant for PMNs and $CD4^+$ cells *in vivo* (Colditz, Watson, 1992; Mulder, Colditz, 1993; Colditz et al. 1994), but is considered to require an intermediate stage of protein synthesis for it's activity (Cybulsky et al. 1989), possibly via the production of other cytokines such as IL-8 (Mulder, Colditz, 1993). This mediator attracts relatively more $CD4^+$ cells than the other mediators used.

The same sheep were subsequently examined for the degree of attenuation of the DTH response.

7.2 MATERIALS AND METHODS

7.2.1 Animals

Six adult female Texel sheep were chosen from the MVV infected flock (2.1, Table 7.1). All of these sheep had been persistently seropositive for MVV, as defined using a standard agar gel immunodiffusion test (2.2). Clinical examination of these sheep revealed no detectable clinical abnormality, and routine haematological samples indicated no abnormality in white blood cell count. Six sex and breed matched control sheep of similar age to the infected ewes were obtained from accredited flocks (2.1, Table 7.1). These control sheep were clinically normal and differential white blood cell counts (2.3) revealed a slight elevation in total white blood cell count in sheep 148 to be the sole abnormality detectable. They were all found to be seronegative for MVV infection at the time of challenge (Appendix 7.2.1).

Table 7.1: Details of the breed, age, sex and serological status of the sheep used in this study

Sheep Number	Status	Breed	Age (years)	Sex
006	MVV +ve	Texel	8	Female
045	MVV +ve	Texel	7	Female
065	MVV +ve	Texel	8	Female
075	MVV +ve	Texel	8	Female
101	MVV +ve	Texel	8	Female
115	MVV +ve	Texel	8	Female
RT05	Control	Texel	5	Female
RT135	Control	Texel	5	Female
RT151	Control	Texel	5	Female
147	Control	Texel	6	Female
148	Control	Texel	5	Female
149	Control	Texel	6	Female

7.2.2 Preparation and source of mediators

ZAP was prepared from plasma extracted from one of the control sheep (149). It was prepared in the manner previously described (Colditz, Movat, 1984; Colditz, Watson, 1992; Mulder, Colditz, 1993; Colditz et al. 1994). Firstly, heparinised blood was collected from the sheep via jugular venepuncture into a sterile container containing endotoxin free heparin (Sigma-Aldrich Company Ltd., Dorset, England). The heparin and blood mixture were then centrifuged at 1500g for 5 minutes, and the resultant supernatant plasma was aseptically decanted. Zymosan extract (Sigma-Aldrich Company Ltd., Dorset, England) was hydrated by boiling in sterile pyrogen free saline for 60 minutes with occasional agitation. The hydrated zymosan was added to the plasma at a rate of 5mg of hydrated zymosan per ml of plasma. This was

incubated for 30 minutes at 37°C, with occasional agitation. The mixture was subsequently centrifuged at 1500g for 5 minutes, and the supernatant was harvested and either used immediately or snap frozen and stored at -70°C until required. The activated plasma was arbitrarily considered to have a concentration of $5x10^{-7}$ mol/l of activated complement component C5a as was assumed by previous authors (Damerau, 1987; Colditz, Watson, 1992; Mulder, Colditz, 1993; Colditz et al. 1994). The ZAP was used at this concentration, and a 0.2ml volume of mediator was injected intradermally into the skin of the medial thigh, equivalent to $1x10^{-10}$ mols of C5a, this quantity having been shown to produce significant accumulation of PMNs and $CD4^+$ cells (Colditz, Watson, 1992; Mulder, Colditz, 1993; Colditz et al. 1994).

Recombinant human interleukin-8 was kindly supplied by Roche Products Ltd., Hertfordshire, U.K.. This was dissolved in pyrogen free phosphate buffered saline (Sigma-Aldrich Company Ltd., Dorset, England) to a dilution such that a 0.2ml injection dose contained $1x10^{-9}$ mols of rh IL8, this quantity having been shown to cause the significant accumulation of PMNs and $CD4^+$ cells (Colditz, Watson, 1992; Mulder, Colditz, 1993; Colditz et al. 1994).

Recombinant ovine tumour necrosis factor α (ro-TNFα) was kindly provided by Mr. Bahram Ebrahimi, Department of Veterinary Pathology, University of Edinburgh, Edinburgh, UK. This was diluted in pyrogen free phosphate buffered saline (Sigma-Aldrich Company Ltd., Dorset, England) such that a 0.2ml injection aliquot contained 0.2ng of ro-TNFα, the quantity that has been shown to attract a significant influx of PMNs and $CD4^+$ cells (Colditz, Watson, 1992; Mulder, Colditz, 1993; Colditz et al. 1994).

Sterile, pyrogen free saline was used as a control injection.

7.2.3 Injection and biopsy technique

The three mediators and control sterile saline were injected into the medial thigh of the sheep, with the precise point of injection marked with black ink (2.4). Biopsies were taken at four hours post injection, this being the time indicated by the previous studies as a time at which a significant infiltration with PMNs and $CD4^+$ cells would have occurred (Colditz, Movat, 1984; Colditz, Watson, 1992; Colditz et al. 1994; Seow et al. 1994). Biopsies were performed in a standard manner (2.6), although an 8mm punch biopsy (Steifel Laboratories Ltd, Buckinghamshire, UK.) was used to collect the skin specimens. The biopsies were bisected and processed in a routine manner (2.6).

7.2.4 Histopathological staining

The fixed specimens were stained in a standard manner with haematoxylin and eosin (2.7.1).

7.2.5 Immunohistological staining

Frozen specimens were sectioned in a cryostat at 6μm, and subsequently stained with SBU-T4 (CD4) monoclonal antibody in a standard fashion as described in chapter 2.

7.2.6 Quantification

Sections were quantified in the manner described by the previous investigators of PMN infiltration (Colditz, Watson, 1992; Mulder, Colditz, 1993; Colditz et al. 1994). This method consisted of the blind counting of the sections using a calibrated eyepiece graticule at x250. Four adjacent fields in the superficial dermis and four adjacent fields in the deep dermis were evaluated, and the total number of PMNs (defined by their morphology) in the four fields of each of these sites was recorded. The four fields counted represented a total counting area of $0.6724mm^2$.

7.2.7 DTH response

The sheep were subjected to an assessment of their DTH response in the standard manner described in 2.4-2.6.

7.3 RESULTS

7.3.1 Gross reaction

Grossly, injection with these mediators produced a relatively small reaction. A reddened firm area of approximately 5mm diameter was visible, although no increase in skin thickness was apparent.

7.3.2 PMN count

PMNs were found at relatively low levels in the control skin biopsies of all the sheep (Appendix 7.3.2). Intradermal injection of all the mediators produced a considerable influx of PMNs, with most of the cells recruited to the deep dermal areas as opposed to the superficial dermal areas (Appendix 7.3.2, Figures 7.1 and 7.2). With all mediators, there was a marked variation in the numbers of cells attracted to the injection sites of the same mediator in different animals (Appendix 7.3.2). The level of PMN cell infiltration was similar in all three mediators. Sites injected with sterile saline as a control exhibited a relatively slight accumulation of PMNs in a small number of the sheep (Appendix 7.3.2).

In all of the biopsies examined, including the control skin and the sterile saline injection site, there were no significant differences in the PMN cell count (i.e. in the superficial dermis, the deep dermis and in the total number counted in both areas) between the MVV seropositive and control sheep (Figures 7.3-7.8), nor were there any significant correlations between the level of PMNs in the mediator injected sites and the size of the subsequently induced DTH reactions.

Figure 7.1

Graph indicating the superficial dermal PMN cell count in skin biopsies at 0 hours and 4 hours post intradermal injection of chemoattractant agents and saline control material in MVV positive and control sheep

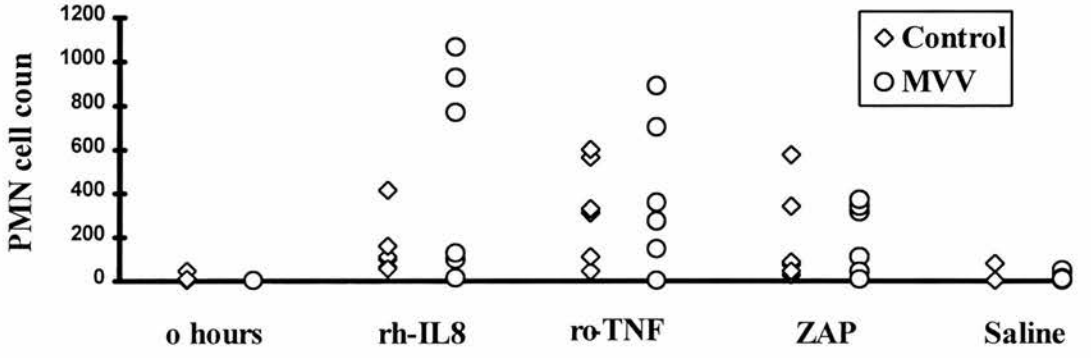


Figure 7.2

Graph indicating the deep dermal PMN cell count in skin biopsies at 0 hours and 4 hours post intradermal injection of chemoattractant agents and saline control material in MVV positive and control sheep

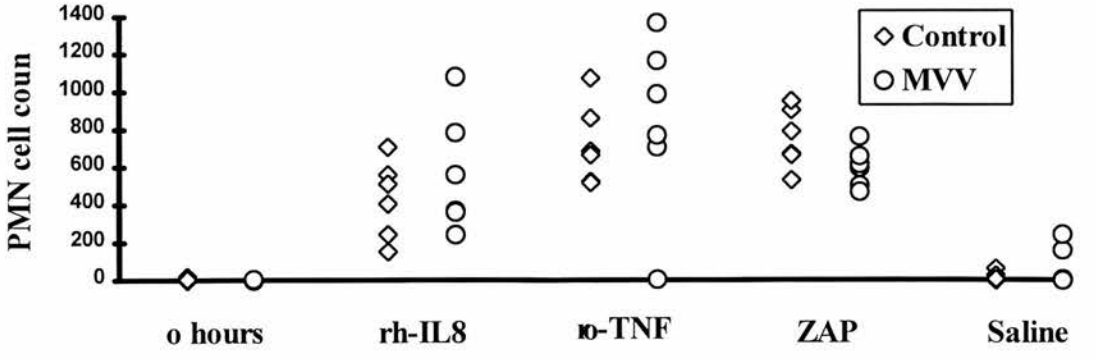


Figure 7.3: Photomicrograph of a biopsy taken from the IL-8 injected site of a control animal. Note the widespread distribution of the infiltrating cells when compared to a PPD reaction. A majority of the infiltrating cells are PMNs (H&E, x250).

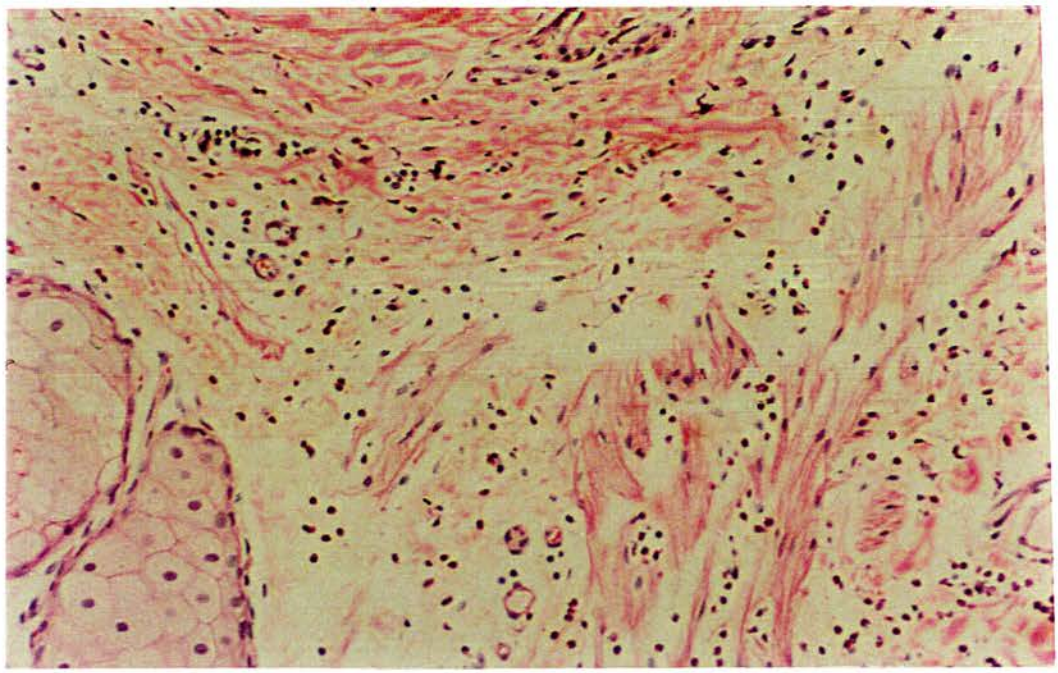


Figure 7.4: Photomicrograph of a biopsy taken from the IL-8 injected site of an MVV infected animal. The distribution and phenotype of the infiltrating cells is very similar to the control specimen (H&E, x250).

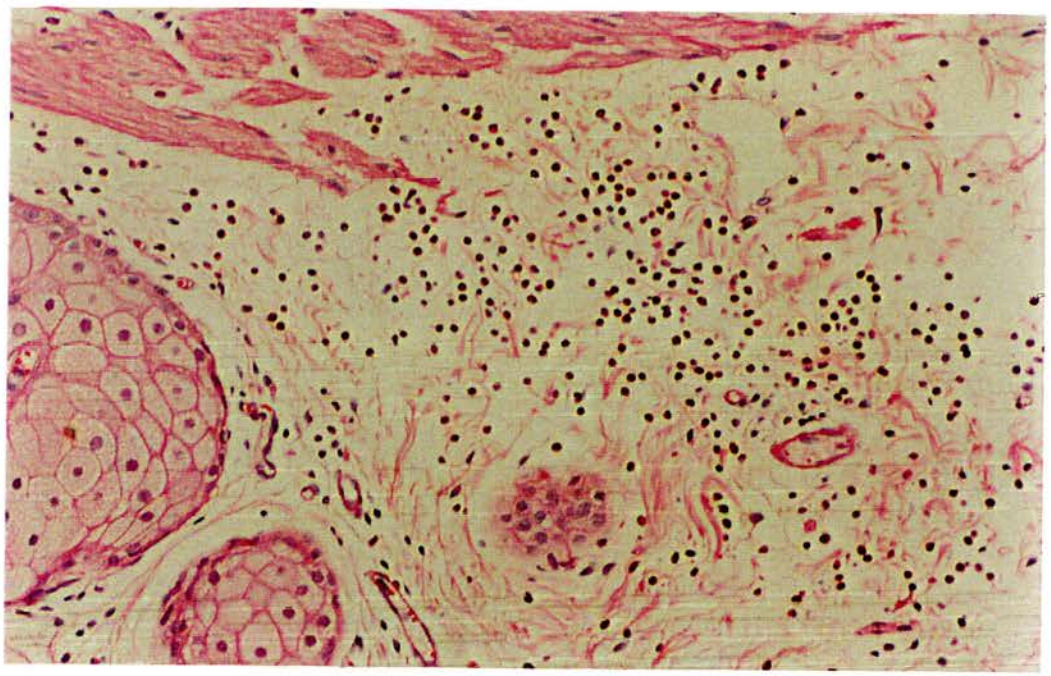


Figure 7.5: Photomicrograph of a biopsy taken from a TNF-α injected site of a control animal. Again, there is a widespread distribution of the infiltrating cells. A majority of the infiltrating cells are PMNs (H&E, x250).

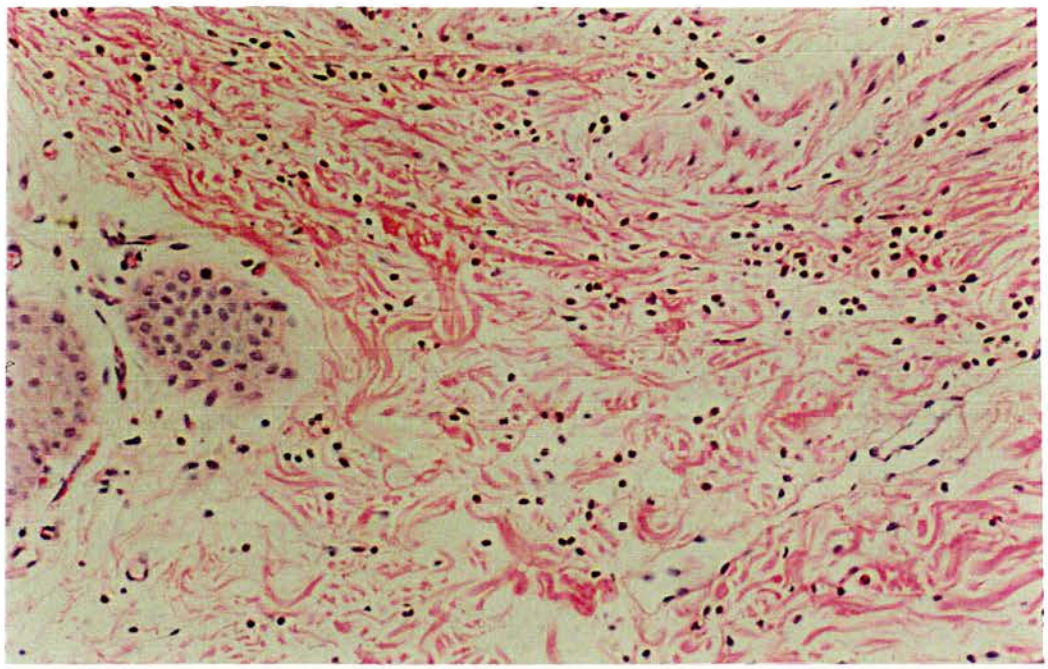


Figure 7.6: Photomicrograph of a biopsy taken from a TNF-α injected site of an MVV infected animal. There is no appreciable difference in the characteristics of the infiltrate (H&E, x250).

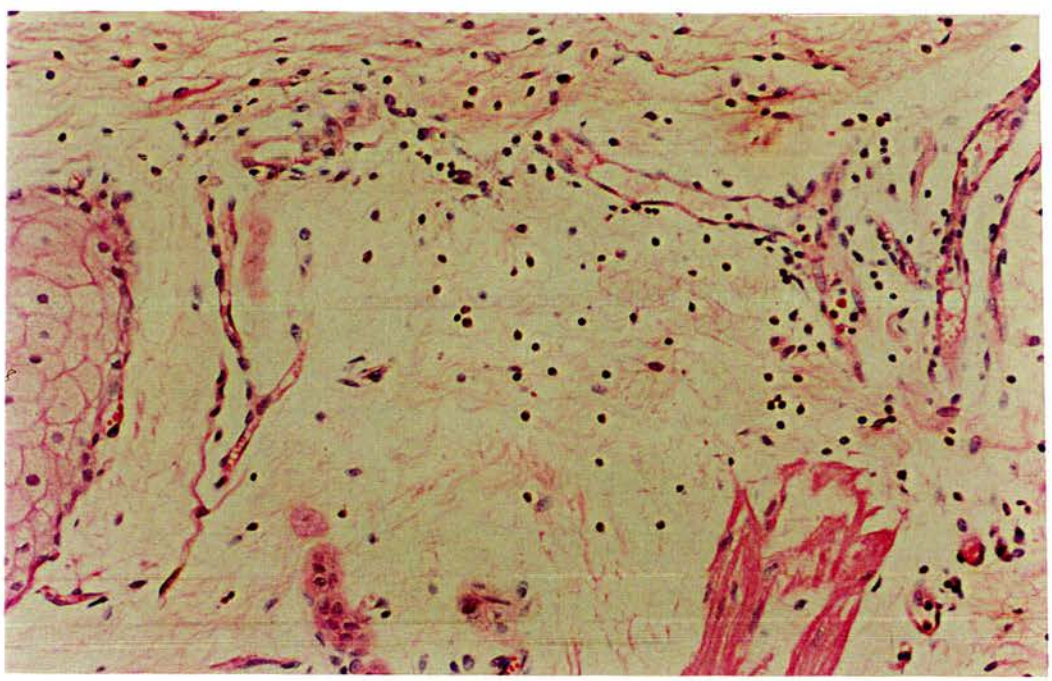


Figure 7.7: Photomicrograph of a biopsy taken from a ZAP injected site of a control animal. A diffuse infiltrate of PMNs is present in the dermis (H&E, x250).

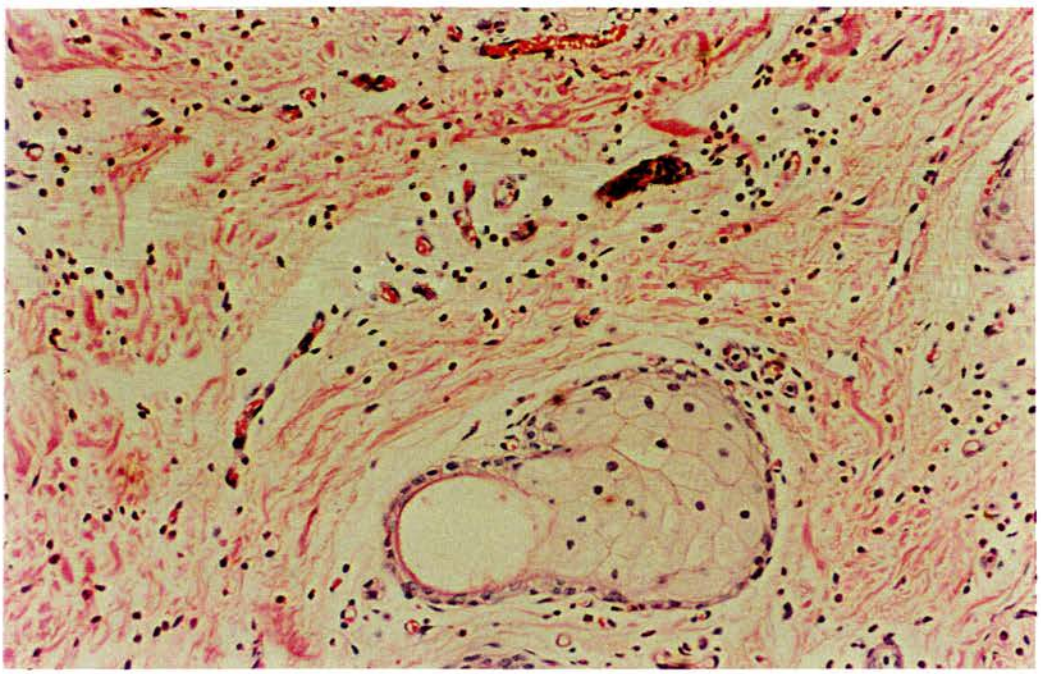
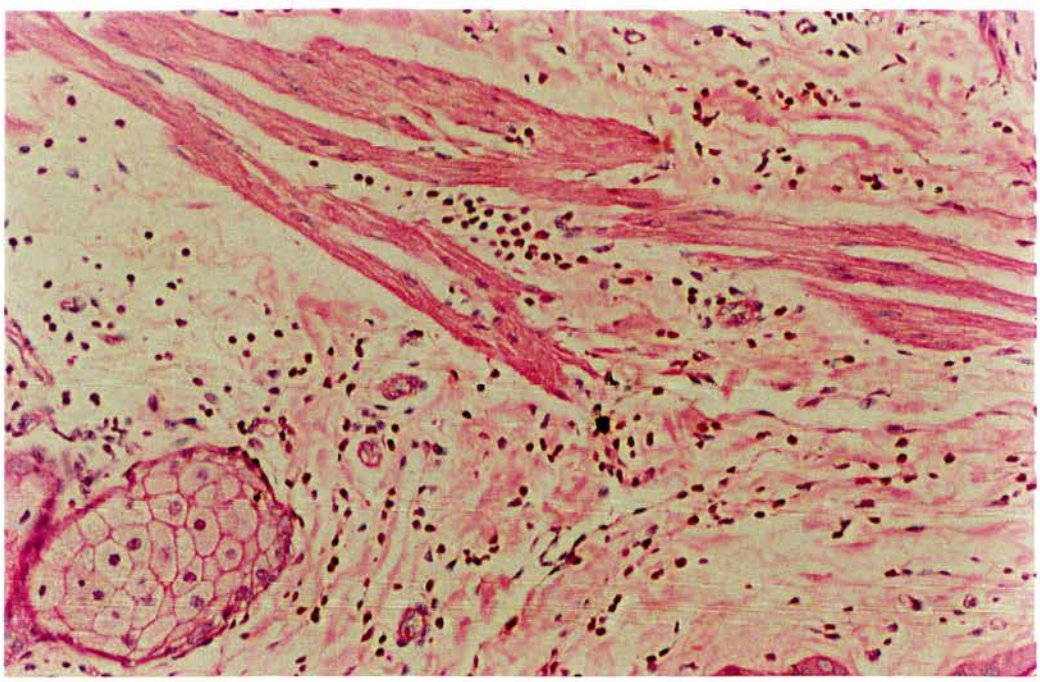


Figure 7.8: Photomicrograph of a biopsy taken from a ZAP injected site of an MVV infected animal. The degree and nature of the infiltrate is indistinguishable from the control sheep (H&E, x250).



7.3.3 $CD4^+$ cell count

$CD4^+$ cells were present in the control skin biopsies taken from all the sheep. Intradermal injection of all mediators produced a relatively low level of migration of $CD4^+$ cells to the site of injection, with TNF-α acting as the most potent attractor of $CD4^+$ cells (Appendix 7.3.3 and Figures 7.9 and 7.10) There was no apparent pattern of distribution between the superficial and deep dermis. Injection of the sterile saline control also produced a marginal influx of $CD4^+$ cells (Appendix 7.3.3 and Figures 7.9 and 7.10).

There were no significant differences in the level of $CD4^+$ (i.e. in the superficial dermis, the deep dermis and in the total number counted in both areas) cells in the control skin or level of $CD4^+$ cell migration produced by any of the injections between the MVV positive and control groups, nor were there any significant correlations between the level of $CD4^+$ cells in the biopsies and the size of the subsequently produced DTH response.

Figure 7.9

Graph indicating the superficial dermal CD4 cell count in skin biopsies at 0 hours and 4 hours post intradermal injection of chemoattractant agents and saline control material in MVV positive and control sheep

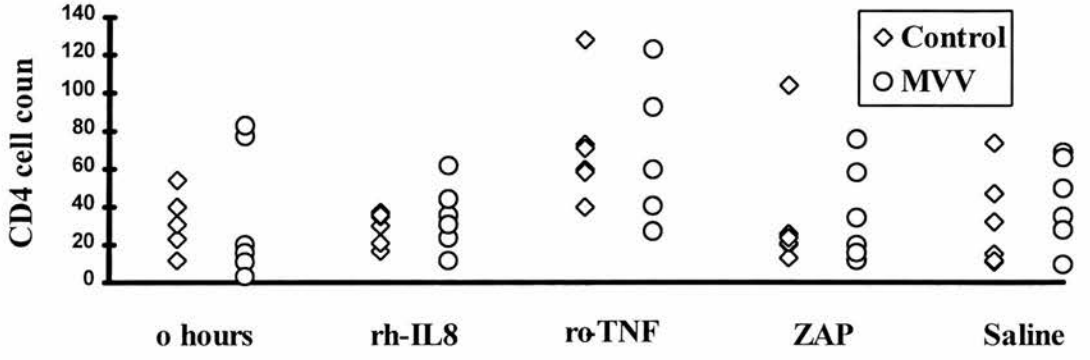
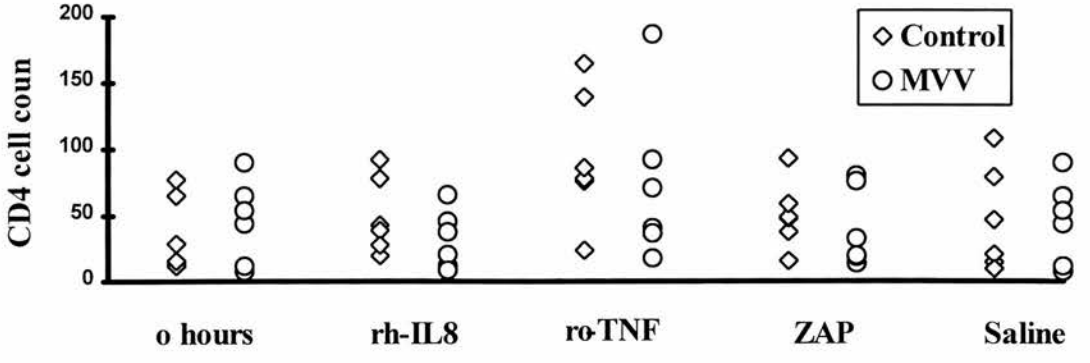


Figure 7.10

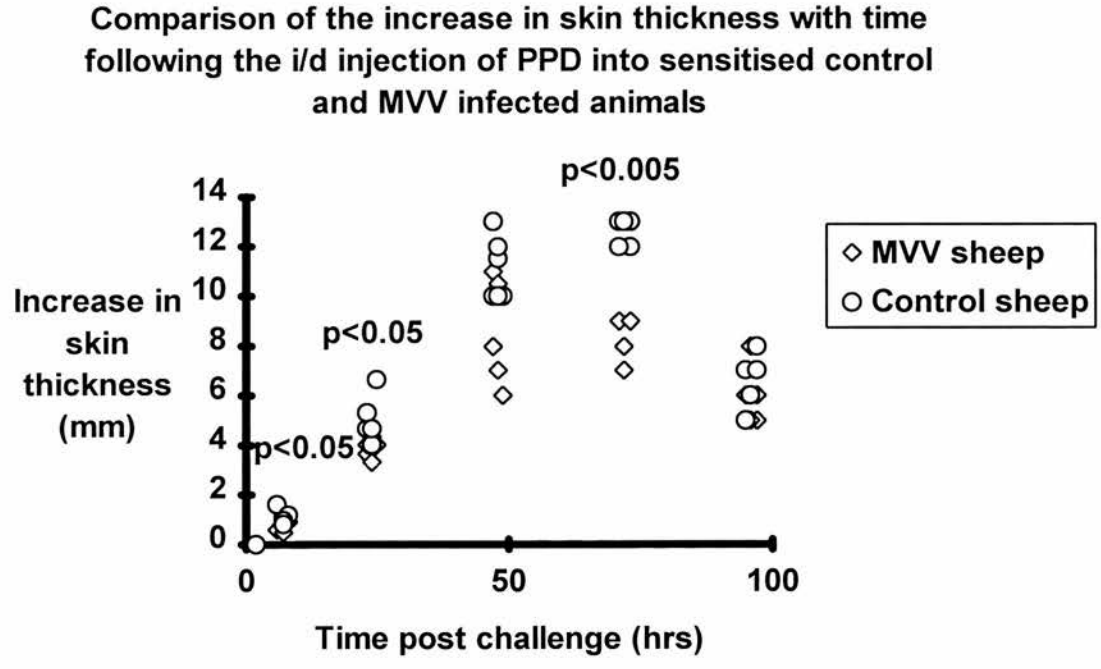
Graph indicating the superficial dermal CD4 cell count in skin biopsies at 0 hours and 4 hours post intradermal injection of chemoattractant agents and saline control material in MVV positive and control sheep



7.3.4 Increase in skin thickness of a DTH response

The DTH responses produced in these sheep were consistent with those previously seen, consisting of a reddened indurative plaque that was maximal at 48-72 hours post injection. The size of the response was significantly depressed in the MVV seropositive animals at 7, 24 and 72 hours post injection (Appendix 7.3.4 and Figure 7.11).

Figure 7.11



7.4 DISCUSSION

The mediators used in this study produced a predominantly PMN dermal infiltration, with a smaller number of $CD4^+$ cells entering the injection site. All mediators produced a similar level of PMN infiltrate, but TNF-α produced a higher level of $CD4^+$ cell infiltrate compared to the other mediators. These findings correspond to the previously published findings (Colditz, Watson, 1992; Colditz et al. 1994; Seow et al. 1994). In this particular study there was a variability in the degree of cell influx induced by the same mediator between individual animals, the previous studies not having presented enough detailed data to allow direct comparison, although the relatively large size of the graphically indicated standard errors of the mean suggest that a marked degree of variability in cell response was present. The results of this study were unable to confirm the previously described preferential accumulation of $CD4^+$ cells in the superficial dermis following TNF-α injection (Colditz et al. 1994), but found individual sheep to have attracted cells preferentially to either the deep or superficial dermis, probably reflecting differences in the depth of the initial dermal injection. This study also produced a more marked degree of cellular accumulation in the control PBS injected sites when compared to the biopsies previously examined in the DTH studies. Since the material and method of injection was identical to the previous experiments, this cellular influx is probably associated with the increased volume of material injected (0.2ml compared to the previous 0.1ml) causing a higher level of tissue damage.

This study found no significant difference in the level of PMN and $CD4^+$ cell influx in response to the intradermal injection of a range of mediators. This provides strong evidence that the lower level of these cells seen in the early DTH response in MVV seropositive sheep is not associated with a decreased ability of these cells to traffic to a site of inflammation. The only comparable studies of PMN migration into induced inflammatory skin sites have centred on the investigation of critically ill

anergic human subjects, where it was found that there was an increased intravascular activation of PMNs associated with a decreased PMN migration into skin blister windows (Tellado, Christou, 1991; Tellado et al. 1993). There is no previously published investigation of the *in vivo* ability of these cell types to traffic to inflammatory foci in any of the related lentiviral infection, although several studies have discovered decreased *in vitro* levels of performance of PMNs from HIV infected individuals (Elbim et al. 1994; Gabrilovich et al. 1994) and SIV infected chimpanzees (Lafrado et al. 1989). Neutropaenia is also recorded in FIV infection (Mandell et al. 1992; Linenberger et al. 1995). The results of the DTH studies indicated that the reduction in size of the DTH response is associated directly with a decreased number of cells in the early response, which suggests that the defect in response is not in the ability of these cells to promote an inflammatory response, but rather in the trafficking of these cells into the lesion. This may be as a result of abnormalities in the endothelial binding, cell chemotaxis into the lesion, or cell retention in the lesion (Nickoloff et al. 1990). The studies of Tellado et al (1991 and 1993) have indicated such a deficiency in PMN traffic in critically ill patients and associated this deficiency with skin test anergy, although the suggestion is that this is due to an abnormality in the PMN cell.

The results obtained by this study indicate that the MVV associated defect in DTH response is not connected with an altered degree of PMN and $CD4^+$ cell traffic to these particular inflammatory signals. This provides evidence that the depression in DTH is associated with a lowered proinflammatory stimulus produced in the early stages of the DTH response, with a lower number of normal cells migrating to this reduced level of signal.

CHAPTER EIGHT

COMPARISON OF ANTI-PPD ANTIBODY PRODUCTION IN CONTROL AND MVV INFECTED ANIMALS

8.1 INTRODUCTION

The previous chapter has shown that the lower density of PMNs and $CD4^+$ cells found in the early DTH lesion of sheep that are seropositive to MVV is not associated with a reduced ability of these cells to migrate to a standard inflammatory stimulus. This finding leads to the suggestion that this deficiency in cell traffic is associated with a relatively lower level of proinflammatory signal produced in the early DTH lesion in the sheep seropositive for MVV.

One of the most potent stimulators of PMN migration is antibody/antigen complex, which causes both the 'classical' activation of complement components and the stimulation of certain cells to produce proinflammatory mediators following the interaction with cell surface Fc receptors, as classically demonstrated in the Arthus reaction (Sylvestre, Ravetch, 1994). The potency of activated complement in attracting PMNs to dermal sites was confirmed in the previous chapter, where it was shown that the intradermal injection of such activated complement components in the sheep induces a marked cellular infiltrate, predominantly PMNs and similar to the infiltrate seen in the early ovine DTH lesion. Indeed, a reaction considered similar in appearance to a classical DTH response has been reported to occur in certain circumstances following the intradermal injection of antigen/antibody complex (Raffel, Newel, 1958). The role of the humoral immune system in the response to both mycobacterial infection and following BCG vaccination has been extensively studied, largely in connection with the development of a diagnostic serological test. This work

has indicated that mycobacterial infection induces an antibody response, although at relatively low levels (reviewed by (Grange, 1984)). There is dispute as to whether BCG vaccination induces a similar antibody response, although one in depth study has suggested that there is an early transitory antibody response following vaccination that appeared at three weeks and declined at seven weeks post vaccination (Diena et al. 1968). This response was primarily of a IgM isotype.

HIV infection has been associated with the increased susceptibility to mycobacterial infections, and it has been shown that in the later stages of the AIDS complex that there is a decrease in the humoral response to mycobacterial PPD components (Barrera et al. 1992).

It was therefore considered conceivable that there may be an antibody involvement in the development of the early DTH response in the experimental BCG vaccination system used, and that the diminished DTH response seen in the sheep seropositive for MVV may be associated with a reduced humoral response to PPD components leading to a reduced influx of PMNs into the early lesion. In order to investigate this possibility, the anti-PPD antibody status in sheep prior to and following BCG vaccination was evaluated in a group of MVV seropositive and matched control sheep.

8.2 MATERIALS AND METHODS

8.2.1 Source of hyperimmune and non immune serum

In order to produce hyperimmune sheep serum to PPD components, a Texel control sheep was hyperimmunised to PPD as described in 2.11.1. Control serum was obtained from blood samples collected from neonatal presuckling lambs as described in 2.11.2.

8.2.2 Animals evaluated

Blood taken from sheep used in a previous DTH experiment (Chapter 5) were assessed for the presence of circulating antibodies (Table 8.1).

Table 8.1: Details of the breed, age, sex and serological status of the sheep used in this study

Sheep Number	Status	Breed	Age (years)	Sex
011	MVV +ve	Texel	9	Female
025	MVV +ve	Texel	9	Female
035	MVV +ve	Texel	8	Female
071	MVV +ve	Texel	9	Female
076	MVV +ve	Texel	8	Female
118	MVV +ve	Texel	8	Female
183	Control	Texel	8	Female
184	Control	Texel	9	Female
245	Control	Texel	8	Female
246	Control	Texel	7	Female
247	Control	Texel	9	Female

8.2.3 ELISA technique

A standard ELISA technique as described in 2.11.3 was employed.

8.2.4 Western blotting technique

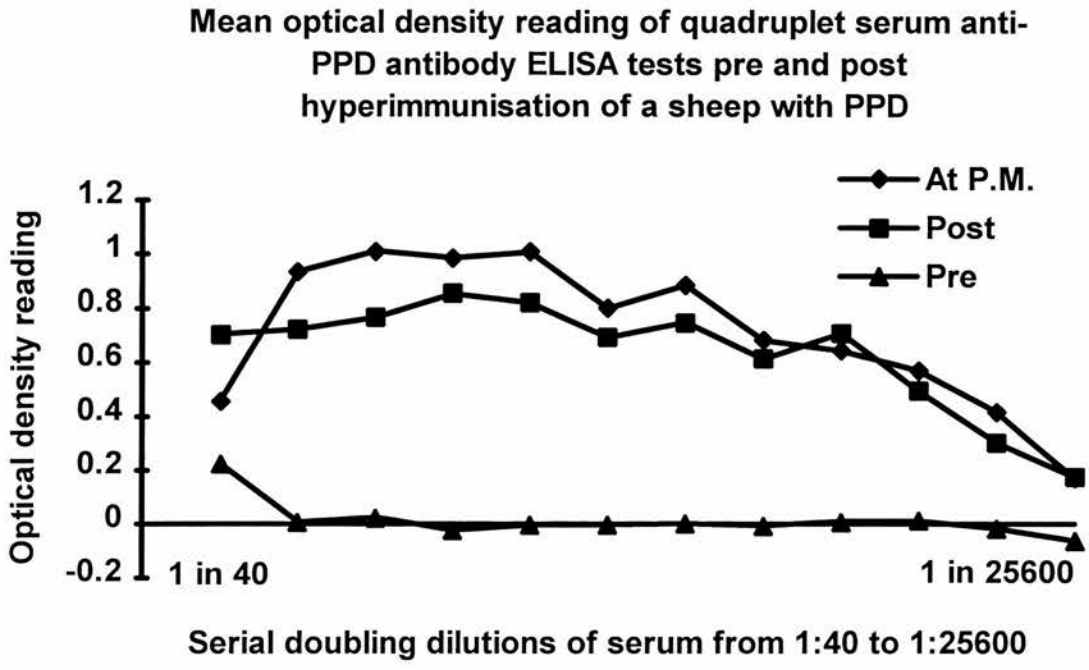
A standard Western blotting technique described in 2.11.4 was employed.

8.3 RESULTS

8.3.1 Optical density readings from the ELISA study

The ELISA technique was first validated using serum samples from the sheep hyperimmunised to PPD. The enzyme reaction was seen to proceed rapidly, and the optical density readings were therefore evaluated following an incubation of 20 minutes at room temperature. A standard serum dilution curve was obtained from this study which enabled the definition of the range of serum dilutions at which there was a linear relationship between the optical density reading and serum dilution (Figure 8.1, Appendix 8.3.1).

Figure 8.1

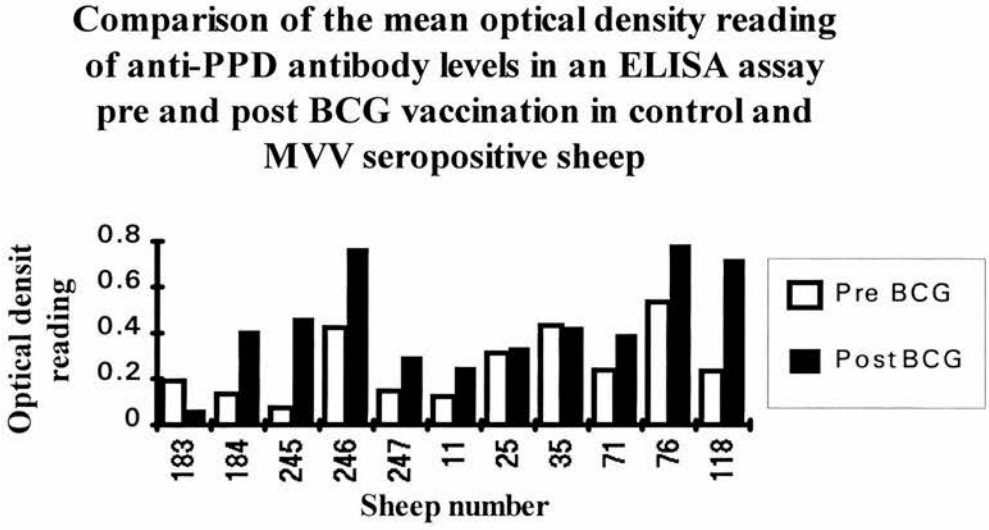


It was discovered that the hyperimmune serum produced a much more intense reaction than the test sera, and that when the test serum was incorporated into the standard ELISA test plates at the concentrations which had been shown to be within the linear area for the hyperimmune serum, there was no apparent reaction in the test wells. The test serum was therefore incorporated into the test ELISAs at higher concentrations such that the serial dilution gave results which were apparent in the optical density readings. The test sera dilutions at which there was a linear relationship between relative dilution and optical density reading were then evaluated and the mean optical density of the duplicate readings at a standard dilution in the centre of this linear area was selected as representative of the quantitative level of anti-PPD antibody and used in the statistical analysis. This standard reading was at a serum dilution of 1 in 40.

The results of this ELISA study indicated a trend of a slight increase in levels of serum anti-PPD antibodies following BCG vaccination, although this was found to be

not significant using a non-parametric Wilcoxon rank test (Figure 8.2 and Appendix 8.3.1).

Figure 8.2



There was a significant negative correlation ($p<0.05$, Spearman ranking correlation) between the optical density reading of the pre vaccinal level of circulating antibodies and the size of the DTH reaction at 72 and 96 hours post challenge following vaccination (Figures 8.3 and 8.4). There were no significant differences between the levels of antibody in the MVV seropositive group and control group either before or after BCG vaccination, and no correlations between the optical density readings and the degree of PMN influx into the DTH lesion. However, there appeared to be a trend towards higher levels of serum anti-PPD antibodies in the MVV seropositive group of animals.

Figure 8.3

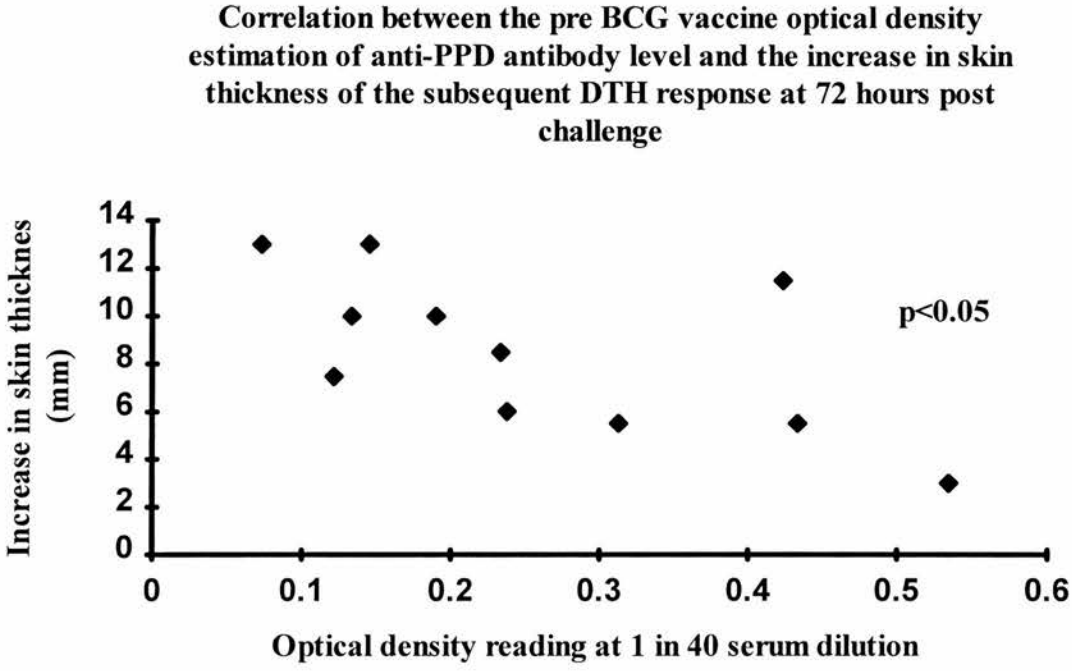
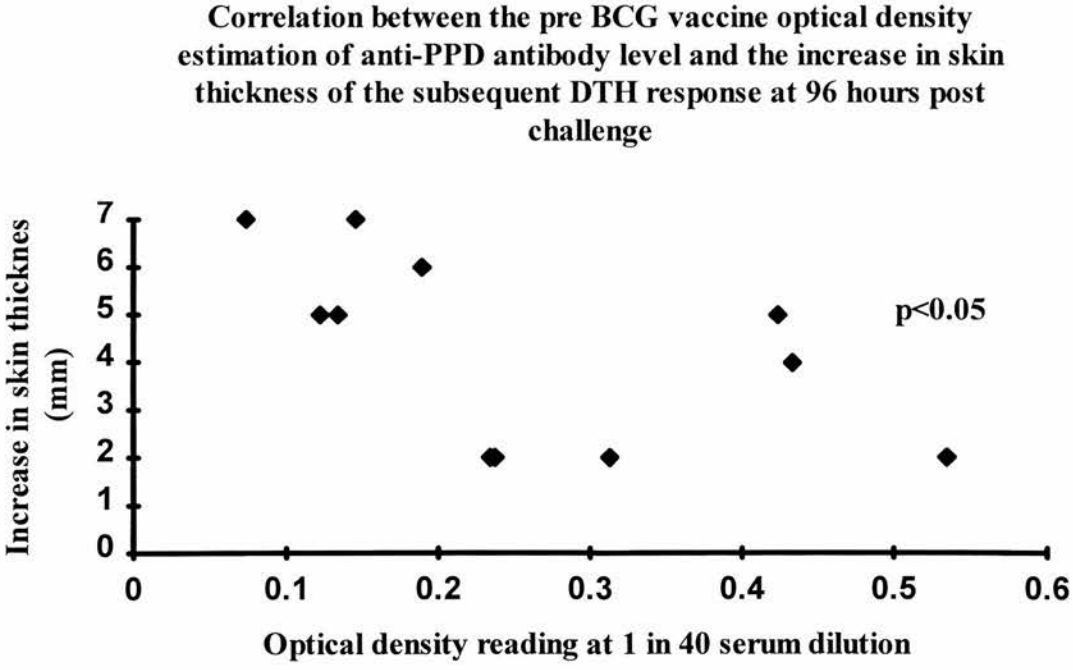


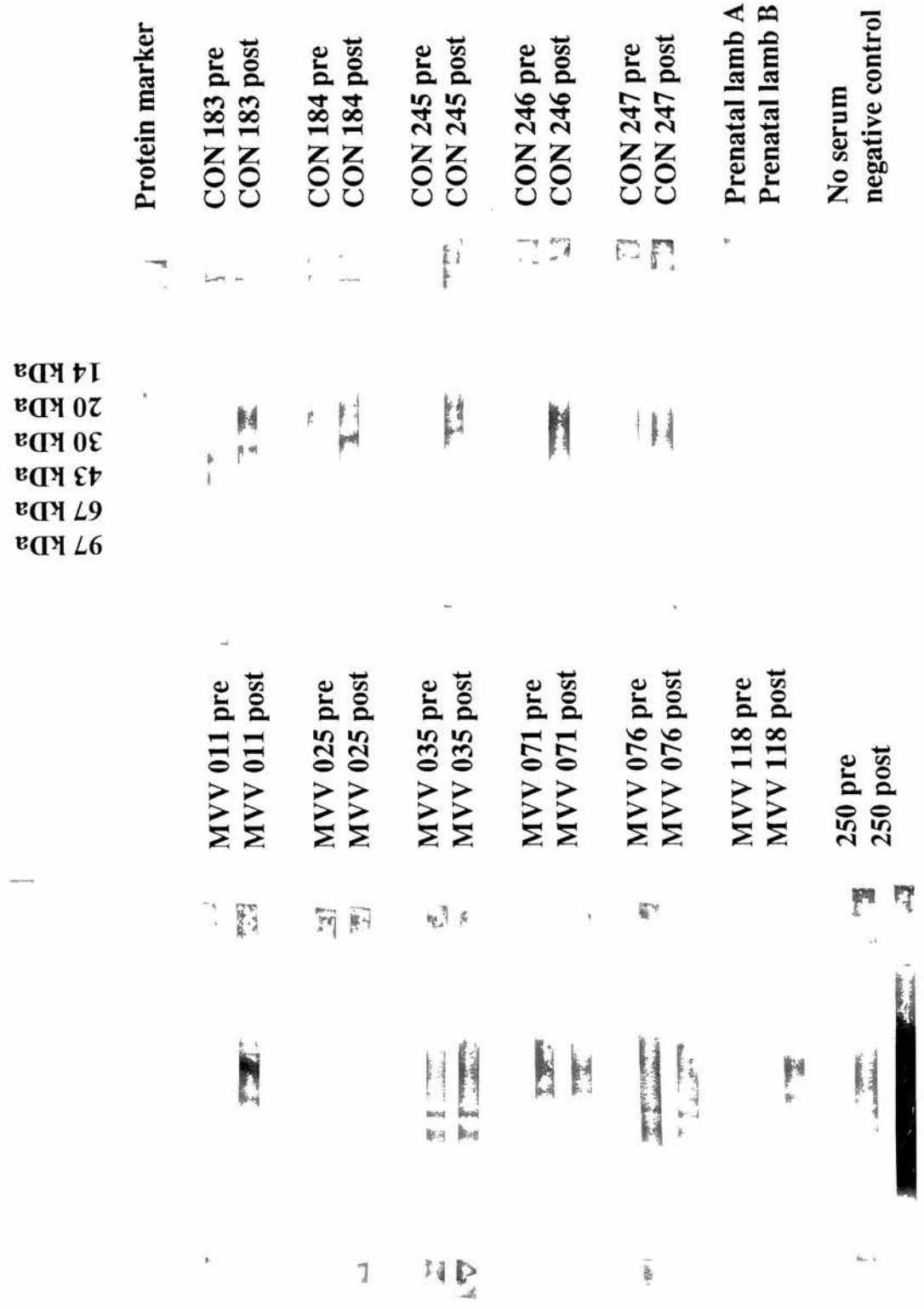
Figure 8.4



8.3.2 Western blotting results

Western blotting of PPD and subsequent staining with amido black protein stain produced a broad smear of staining throughout the length of the blot, with no distinct bands visible, confirming the diverse and complex protein composition of PPD. Development of the blotted PPD strips with both PBS and prenatal lamb serum produced a faint banding pattern, indicating the non-specific binding of the secondary anti-sheep IgG antiserum to components of the PPD. The sheep hyperimmunised to PPD exhibited a marked increase in antibody binding, although the pattern was one of an intense broad band of reactivity throughout the whole protein spectrum, with no distinct banding patterns visible. In comparison, the pre and post vaccinal serum samples from both the MVV seropositive and control group of animals exhibited relatively low levels of antibody binding, with many animals having apparently similar levels of reaction as the negative controls, suggesting that the level of circulating anti-PPD antibody was negligible in these animals. In the sera which did have an apparent increase in antibody levels following BCG vaccination, the increase in antibody binding occurred in the area of the lower molecular weight markers of approximately 14-20 kilodaltons (Figure 8.5), with the binding occurring as a smear in most instances, although in some situations specific bands were noticeable. Subjectively, there was no apparent difference in antibody binding between the two groups of sheep, either before or after BCG vaccination, and there was no apparent difference in the response to BCG vaccination. There appeared to be a trend towards more marked banding patterns in the MVV seropositive group of sheep (Figure 8.5).

Figure 8.5: Photograph of the developed Western blot strips illustrating the relatively weak banding pattern in the samples when compared to the positive and negative controls. A slight increase in band intensity is noticeable following vaccination in some samples. There is a trend towards stronger bands in the MVV infected sheep samples.



When comparing the results obtained using the different methods of quantifying antibody levels, there were similarities noted between the relative change in optical density reading in the ELISA and the subjective alteration in density of the antibody bands in the Western blots in the same animal assessed pre and post BCG vaccination, although there appeared to be much less correlation between the ELISA readings and the density of the Western blot bands between individual animals.

8.4 DISCUSSION

The results of this experiment have shown that there is no demonstrable difference in the levels of circulating anti-PPD antibodies between the group of sheep seropositive to MVV and the control sheep when assayed quantitatively in an ELISA assay, with all levels being relatively low when compared to the serum of a sheep hyperimmunised to PPD. There appeared to be a trend towards higher levels of serum antibodies in the MVV seropositive group of animals both prior to and following BCG vaccination. There was a significant negative correlation between the pre vaccinal antibody optical density readings and the later increase in skin thickness of a DTH response. There was no correlation between post vaccinal antibody optical density readings and increase in skin thickness in the DTH response. There was also no significant correlation between the levels of circulating antibodies and the degree of influx of PMNs into the DTH lesion. When the same serum samples were studied subjectively in the Western blots, there were no marked differences between the groups, although there was a slight trend towards higher levels in the MVV seropositive group, which would suggest a negative correlation between the antibody level and the degree of PMN influx into and increase in skin thickness in the DTH response, although this finding requires more detailed investigation.

The small differences in results obtained by the two different methods probably result from the alterations in antigen characteristics that occur following SDS

treatment and linearisation, and also reflects the minimal levels of antibodies circulating in the serum of these sheep either prior to or following BCG vaccination.

This work has confirmed previous investigations in other species (Grange, 1984; Ivanyi et al. 1988; Fifis et al. 1994), showing that the humoral component of the immune system plays a relatively minor role in the immune response to BCG vaccination, as judged by an increase in circulating levels of antibodies to mycobacterial PPD. The finding of a significant negative correlation between the pre vaccinal levels of circulating antibodies and the eventual maximal size of the DTH would provide evidence that antibodies are in fact detrimental to the development of a DTH response, although the significant correlation was no longer present when the DTH was initiated following the administration of BCG vaccine. This latter finding probably reflects the relatively marginal levels of circulating antibodies both pre and post vaccination. Studies in cattle have also suggested that there is a reciprocal relationship between the levels of antibodies and development of a PPD initiated DTH in cattle affected with M. tuberculosis (Plackett et al. 1989). Indeed one study has suggested that the presence of high levels of antibodies directly suppress the DTH response (Morikawa et al. 1991). The inverse relationship between antibody levels and DTH response size and the subjectively higher levels of circulating antibodies found in sheep seropositive to MVV may indicate an inappropriate stimulation of the humoral immune system as oppose to the cell mediated immune response in the MVV sheep. This change of emphasis towards the humoral side of the immune system is considered to be a feature of the later stages of HIV infection, where it is linked to a change in the relative population of Th1 and Th2 cells, with an increased proportion of Th2 cells and decreased proportion of Th1 cells. This change in turn leads to a predominantly Th2 type response resulting in a relative increase in antibody production and lowering of the cell mediated immune response (Meyaard et al. 1993). Whilst the existence of these two Th subsets is not known in the sheep, it is interesting to speculate that this mechanism may be involved in the depression of the DTH.

In conclusion, the experimental work in this chapter has shown that there appears to be little involvement of the humoral immune system in response to BCG vaccination, and that there is no significant difference in the level of circulating anti-PPD antibodies between the MVV seropositive group and the control animals at any stage in the DTH experiment. There was, however, an inverse relationship between the levels of prevaccinal antibodies and the development of a gross DTH lesion, although there was no significant inverse association between the level of circulating antibody and the recruitment of PMNs to the site of the DTH lesion. These findings would suggest that antibodies play little part in the recruitment of PMNs to the ovine DTH lesion, and that the deficiency in this process in sheep seropositive to MVV is not related to alterations in the levels of such circulating antibodies. These findings would, however, not rule out the possibility that there is a change of emphasis towards a humoral response and away from a cell mediated response in the sheep seropositive to MVV, analogous to the situation in people infected with HIV. Speculatively, this change in emphasis may be associated with a change in relative levels of Th1/Th2 cells, and with the resultant alteration in cytokine production profiles.

CHAPTER NINE

COMPARISON OF THE MRNA PRODUCTION PROFILE IN THE DTH LESION OF MVV SEROPOSITIVE AND CONTROL SHEEP

9.1 INTRODUCTION

The preceding work has indicated that the defect in PMN and $CD4^+$ cell migration into a DTH reaction in MVV seropositive sheep appears to be associated with an alteration in the proinflammatory processes occurring at an early time point in the reaction. Circulating anti-PPD antibody levels have been shown to be inversely related to the development of the DTH response, indicating that antibody/antigen complexes are not involved in the inflammatory process. The finding of a higher level of circulating antibodies in the MVV infected sheep provided limited evidence that these sheep have a tendency towards a humoral immune response, and the finding of a depression in DTH suggested that MVV infected sheep had a tendency for a reduced cell-mediated immune response. These findings combined would correlate to suggest a hypothesis of an MVV induced switch from a Th1 type response to a Th2 type response analogous to that found in HIV infection (Meyaard et al. 1993).

Previous studies of inflammatory mediator involvement in PPD induced dermal DTH responses have indicated that cytokines are crucial to the development of the lesion. In particular, there is evidence for the production of predominantly Th1 type cytokines, with the specific importance of IFN-γ in the ovine model being indicated by the experimental reduction in DTH size achieved by treatment with an anti-IFN-γ neutralising monoclonal antibody (Emery, Davey, 1995). Further work has confirmed the abundant presence of IFN-γ in the later (post 24 hour) DTH reaction, along with the other main Th1 type cytokine IL-2, and has also indicated an considerable level of

TNF-α (Chu et al. 1992; Tsicopoulos et al. 1992; Tsicopoulos et al. 1994; Chensue et al. 1994; Ng et al. 1995). These studies reported a relatively low level of expression of main Th2 type cytokine IL-4. One investigation reported a high level of expression of IL-10 mRNA in the DTH (Ng et al. 1995). This cytokine is reported as exerting a general suppressive effect on Th1 type responses and is considered to be involved in the cross regulation between Th1/Th2 lymphocyte subsets (Mosmann, 1994). IL-10 has also been reported to depress the production of other proinflammatory cytokine production and reduce dermal swelling when administered immediately prior to induction of a DTH type response (Li et al. 1994).

This information suggests that there could be an alteration of the cytokine profile in the DTH response of the MVV seropositive sheep, with either a decrease in the induced expression of a proinflammatory cytokine(s) or an increase in the constitutive or induced expression of anti-inflammatory cytokines. It was therefore important to assess the presence of a range of these cytokines in the early response of both an MVV seropositive and control group of sheep. Since there was already a significant difference in the density of inflammatory cells within the lesion by 24 hours post antigen injection, and PMN margination and migration had also been shown to have commenced by 7 hours, it was decided to assess the cytokine profile in biopsies taken at 0 and 7 hours post PPD and PBS injection, and compare these with similar samples taken from control sheep.

Since there were relatively few cells in these early biopsies, a sensitive technique was required to undertake these investigations, and RT-PCR technology was therefore employed in order to assess the production of cytokine mRNA in the DTH lesions (Arnheim, Erlich, 1992). In order to further increase sensitivity and to show specificity, the PCR products were Southern blotted and probed using radiolabelled internal sequence oligonucleotide probes. The cytokines whose expression was investigated were those that had previously been shown to be

important in both the induction and suppression of DTH responses, concentrating on those that had been shown to induce dermal migration of PMNs and lymphocytes when injected intradermally in sheep. IFN-γ is a Th1 cytokine that has been reported to be crucial to the development of the DTH lesion (Tsicopoulos et al. 1992; Tsicopoulos et al. 1994; Chensue et al. 1994; Ohmen et al. 1995; Emery, Davey, 1995; Ng et al. 1995), and has also been shown to induce significant accumulation of lymphocytes and PMNs when injected dermally in sheep(Colditz, Watson, 1992). IL-2 is similarly a Th1 type cytokine, and is expressed in DTH lesions(Tsicopoulos et al. 1992; Tsicopoulos et al. 1994; Ng et al. 1995). In order to assess the level of IL-2 in the lesions, the expression of mRNA for the IL-2R was examined, as this receptor molecule is expressed in response to IL-2 mediated activation. Expression of IL2R is therefore considered to be a marker for the IL-2 mediated activation of T cells (Budjoso et al. 1992). TNF-α involvement has also been reported in DTH lesions (Ng et al. 1995), and has also been shown to induce significant traffic of PMN and lymphocytes when injected dermally in sheep (Colditz, Watson, 1992) Finally, IL-10 expression was also investigated as it has been shown to occur in DTH responses (Ng et al. 1995), and may exert a suppressive effect (Li et al. 1994) analogous to the effect associated with MVV seropositivity. The opportunity was also taken to use RT-PCR to evaluate the presence of viral RNA in the skin sample in order to see whether there was a direct correlation between the presence of virus and the suppression of DTH.

9.2 MATERIALS AND METHODS

9.2.1 Animals used

Six adult female Texel sheep were selected from the MVV infected flock (2.1, Table 9.1). All of these sheep had exhibited a persistent serological response to MVV, as defined using a standard agar gel immunodiffusion test. Six sex and breed matched control sheep of similar age to the infected ewes were obtained from accredited flocks (2.1, Table 9.1). These control sheep were clinically normal and serologically free from MVV infection at the time of challenge.

Table 9.1: Details of the sheep used in this experiment

Sheep Number	Status	Breed	Age (years)	Sex
064	MVV +ve	TexelX	9	Female
103	MVV +ve	Texel	>10	Female
155	MVV +ve	TexelX	4	Female
168	MVV +ve	Texel	4	Female
174	MVV +ve	Texel X	4	Female
255	MVV +ve	Texel	4	Female
51	Control	Texel	2	Female
52	Control	Texel	5	Female
54	Control	Texel	5	Female
55	Control	Texel	4	Female
56	Control	Texel	2	Female
57	Control	Texel	2	Female

9.2.2 Initiation, measurement and biopsy of the DTH

The DTH was initiated, measured, and biopsied in the standard manner described in 2.4 - 2.6. Biopsies were taken initially from untreated skin and from PPD and PBS injected sites at 7 hours post injection. Biopsies were immediately wrapped in aluminium foil and snap frozen in liquid nitrogen in order to preserve the integrity of the RNA. Samples were maintained at -70°C for as short a time as possible before the RNA was extracted.

9.2.3 Isolation of RNA

RNA was extracted from the tissue using a commercial kit as described in 2.12.2. Reverse transcription was performed as outlined in 2.13.1.

9.2.4 PCR technique

The PCR was performed as described in 2.13.2. Positive control material was provided using specific plasmid cloned DNA sequences. In order to assess that RNA isolation and reverse transcription had been performed successfully, the presence of mRNA for the ubiquitous 'reporter' mRNA ATPase was first verified with the PCR (Woodall et al. 1994). Samples were then subjected to PCR for specific cytokine messages. The sequences of the primers and probes used is listed in Appendix 9.2.4.

9.2.5 Radiolabelled probing

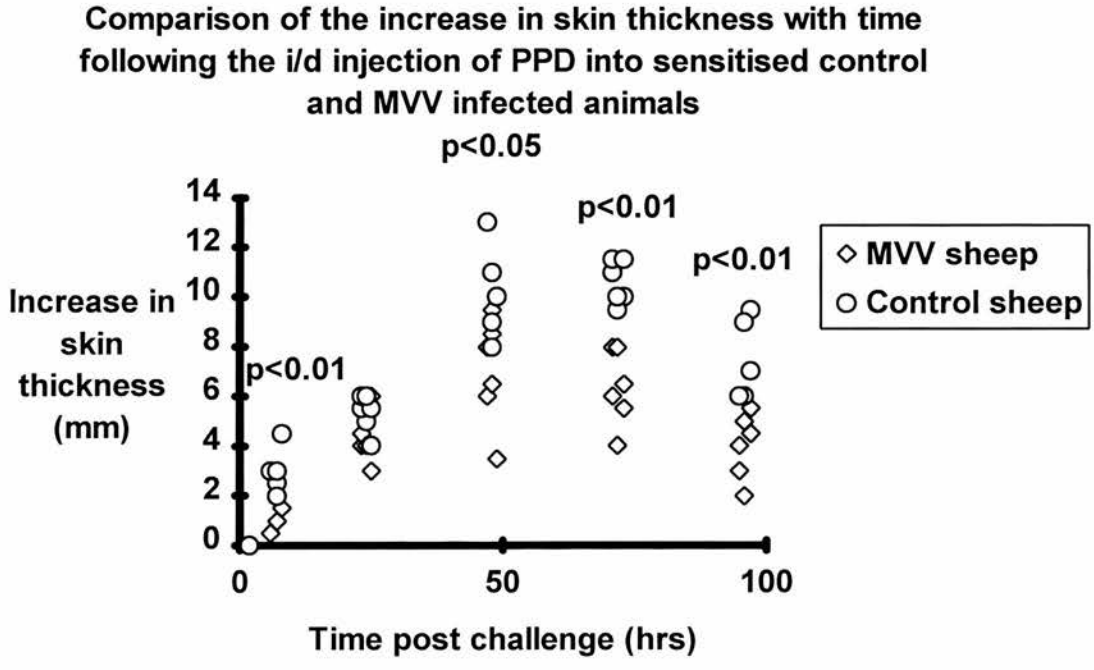
Radiolabelled probing was performed using probes complementary for internal sequences of the PCR product as described in 2.14.2. Probed blots were left in contact with X-ray film for variable lengths of time dependent upon the level of radioactivity detected on the blot using a Geiger-Muller counter. This ranged from 3 hours at room temperature for blots with high levels of radioactivity to 6 days at -70°C for blots with minimal levels of radioactivity. Probe sequences are listed in Appendix 9.2.4.

9.3 RESULTS

9.3.1 Skin thickness

In accordance with previous results, increases in skin thicknesses following PPD injection were maximal at 48-72 hours post injection (Figure 9.1, Appendix 9.3.1). There was a significant depression in the increase in skin thickness seen in the MVV seropositive sheep as indicated in Figure 1. No swelling was observed at the site of the PBS injection.

Figure 9.1



9.3.2 ATPase PCR

ATPase PCR products were detected in all the samples with the exception of those from the sheep 56 PPD biopsy and the sheep 64 PBS biopsy. This confirmed that mRNA had been successfully isolated and reverse transcribed from 34 of the 36 biopsy specimens (Appendix 9.3.2 and Figure 9.2).

9.3.3 Virus PCR

The presence of virus was investigated using primers and probe specific for the Pol region of the genome. The particular primer set and probe combination had been shown to reliably detect a large range of virus isolates (Woodall and Ebrahimi, personal communication). The positive control lanes produced marked bands on the autoradiographs. All other lanes were negative (Appendix 9.3.2 and Figure 9.3).

9.3.4 IFN-γ PCR

The autoradiograph of the probed blot of the IFN-γ PCR products exhibited strong bands in the positive control lanes. Otherwise, IFN-γ was rarely detected in any of the sample lanes, being present as a relatively weak signal in samples from the following biopsies: 51 control, 54 PBS, 55 control (Appendix 9.3.2 and Figure 9.4). There was no apparent difference in distribution between the two groups of sheep.

9.3.5 IL-2R PCR

The autoradiograph of the probed blot of the IL2-R PCR products exhibited strong bands in the positive control lanes. Again, IL2-R was rarely detected in the sample lanes with a moderate signal present in the sample from biopsy 51 PPD, and weak signals present in samples from the following biopsies: 52 PBS, 155 PPD, 064PPD, 168 PBS, and 255 PBS (Appendix 9.3.2 and Figure 9.5). There was no apparent difference in distribution between the two groups of sheep.

9.3.6 TNF-α PCR

The autoradiograph of the probed blot of the TNF-α PCR products exhibited only a weak band in the positive control lanes. In the sample lanes,a moderate positive signal was detected in the sample from 057 PPD, and weak positive signals were detected in a wide range of samples, including control, PBS and PPD biopsies (Appendix 9.3.2 and Figure 9.6). There was no apparent difference in distribution between the two groups of sheep.

9.3.7 IL-10 PCR

The autoradiograph of the probed blot of the IL-10 PCR products exhibited strong bands in the positive control lanes. In the sample lanes, strong positive signals were detected in a wide range of samples, including control, PBS and PPD biopsies. There were no clear distinctions between the groups in the expression of IL-10 mRNA, although there was a trend towards stronger bands in the PPD biopsies of the MVV group (although it should be stressed that this was not a quantitative experimental system) (Appendix 9.3.2 and Figure 9.7).

Figure 9.2: Autoradiograph of the internally probed ATPase RT-PCR reaction products. Bands are present in all lanes except 56 PPD and 64 PBS indicating successful isolation from a majority of samples.

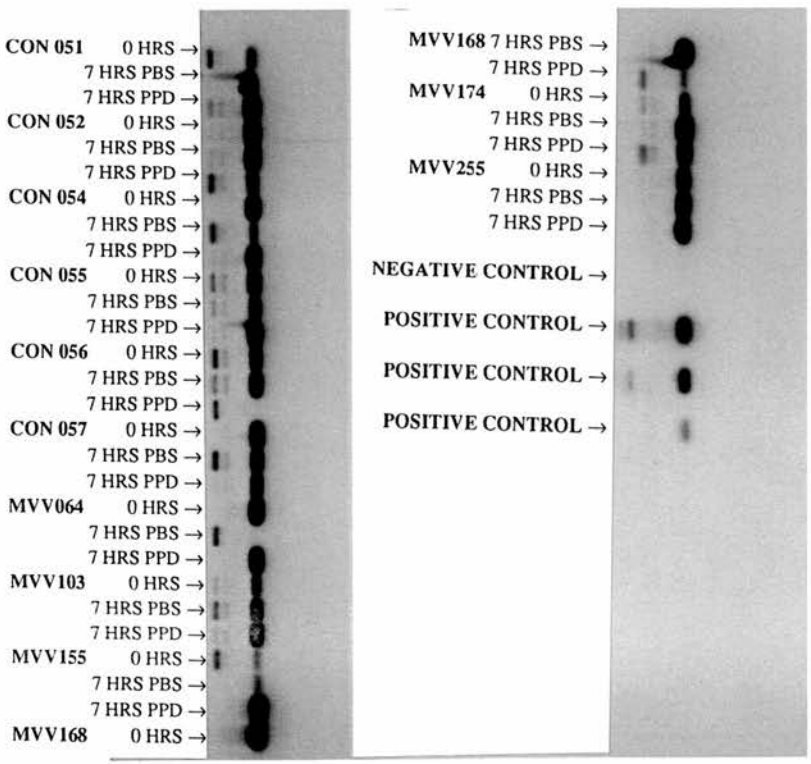


Figure 9.3: Autoradiograph of the internally probed MVV pol 2 RT-PCR reaction products. Bands are only present in the positive control lanes. The mark near 103 PBS is an artefact.

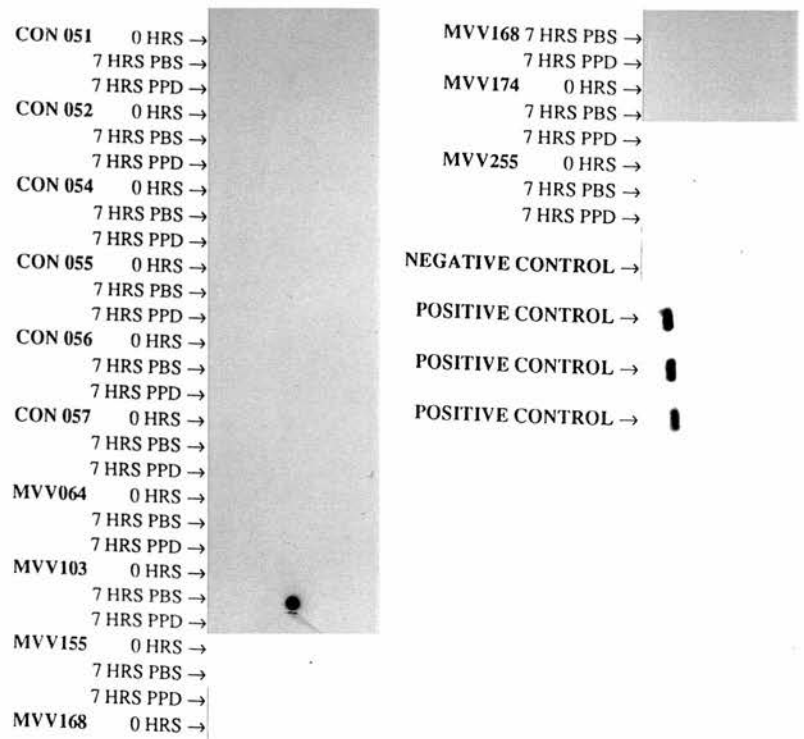


Figure 9.4: Autoradiograph of the internally probed IFN-γ RT-PCR reaction products. There are weak positive bands in a few samples.

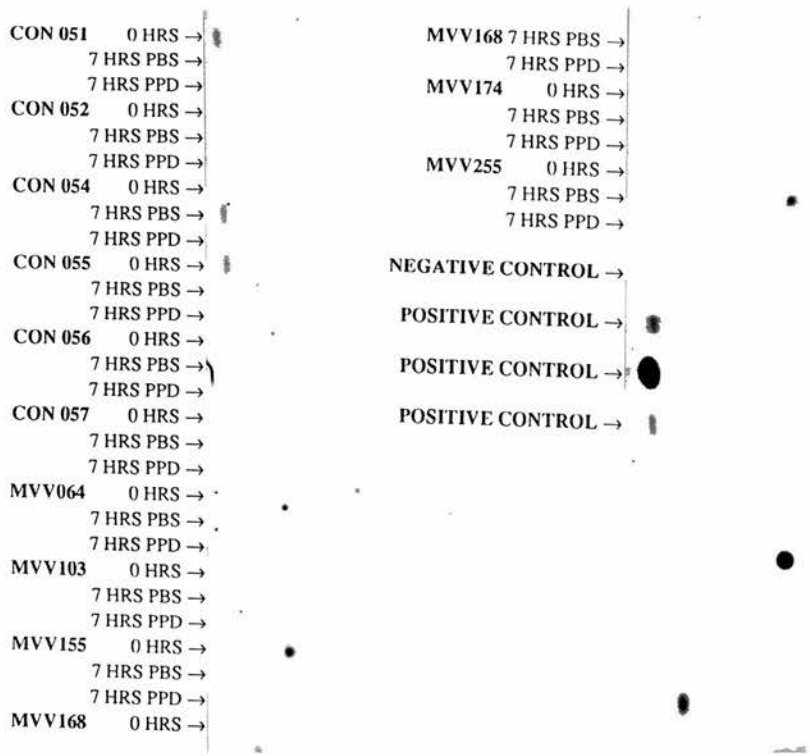


Figure 9.5: Autoradiograph of the internally probed IL2R RT-PCR reaction products. Again, there are relatively few bands in the sample lanes.

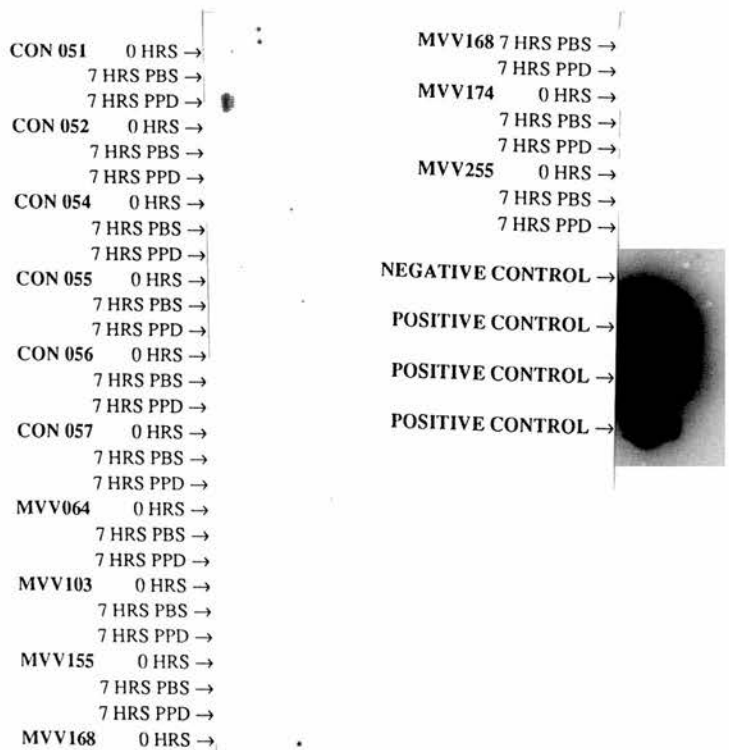


Figure 9.6: Autoradiograph of the internally probed TNF-α RT-PCR reaction products. Weak bands are present in a large number of the sample lanes, although there is no discernible pattern of mRNA production.

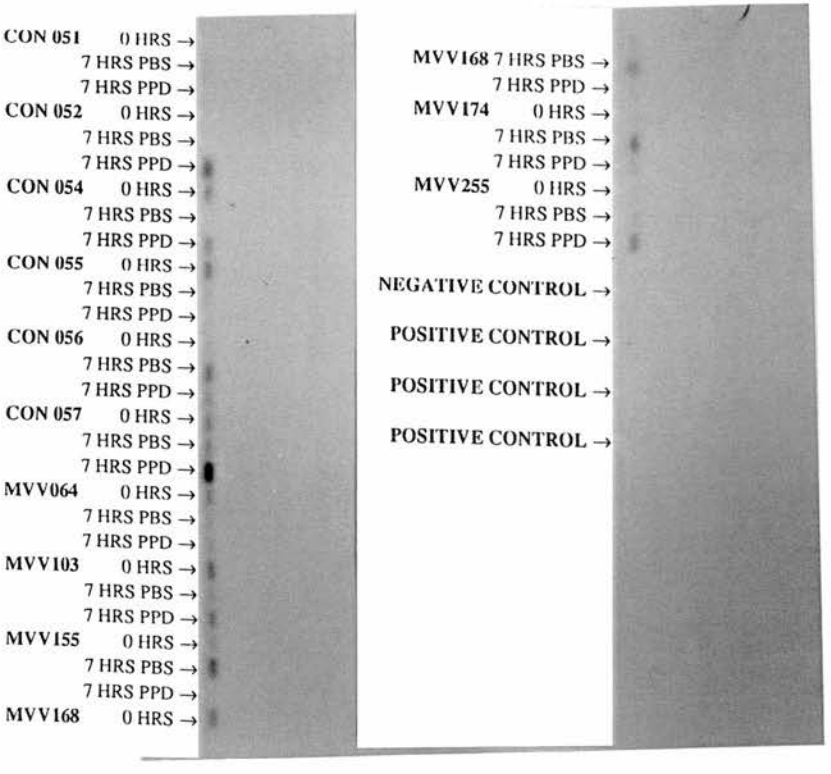
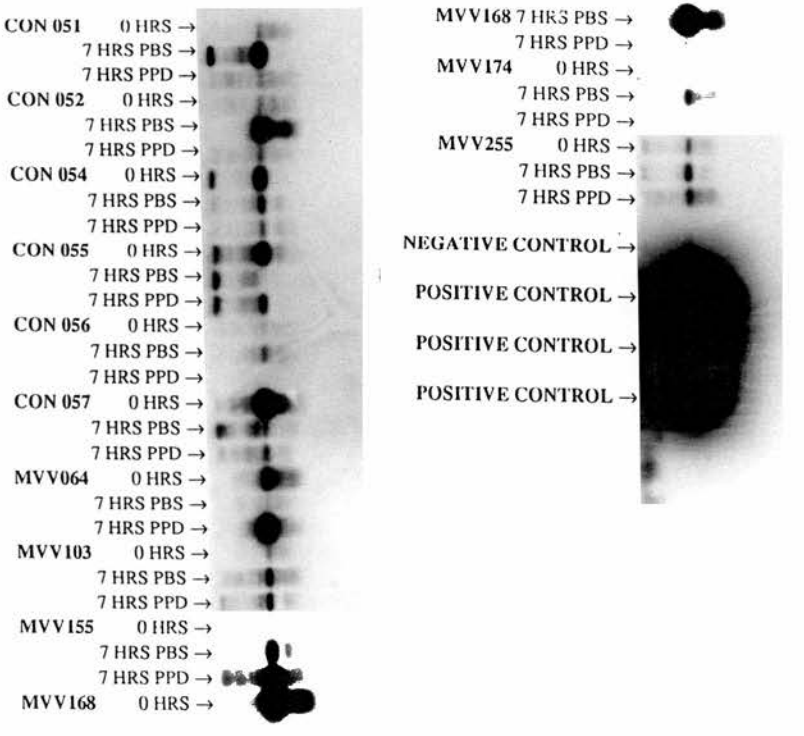


Figure 9.7: Autoradiograph of the internally probed IL10 RT-PCR reaction products. Strong bands are present in many of the sample lanes. There is a trend towards stronger banding in the MVV PPD samples.



9.4 DISCUSSION

The results of this study indicate that there is no apparent alteration in the cytokine mRNA profile in the early DTH reaction in sheep seropositive to MVV that exhibit depression in the maximal size of the response. Furthermore, MVV specific RNA was not present in the skin biopsies removed from the MVV group of sheep, mRNAs coding for the proinflammatory cytokines IFN-γ and TNF-α and proinflammatory indicator IL2-R were not consistently present in the biopsies, with many samples being negative, and there was a marked expression of IL-10 mRNA in a large number of the biopsies, with a trend towards stronger banding in the MVV infected sheep.

Previous studies of the expression of cytokine mRNAs in the PPD initiated DTH response have utilised murine, rattine, bovine and human subjects; and have largely concentrated on time points of 24 hours or later. These studies have consistently reported the presence of IL-2 and IFN-γ in the DTH at 24-72 hours using immunohistochemistry, in situ hybridisation and RT-PCR techniques (Chu et al. 1992; Tsicopoulos et al. 1992; Chensue et al. 1994; Ohmen et al. 1995; Tsicopoulos et al. 1994; Ng et al. 1995), with one study indicating a positive immunohistochemical staining for IFN-γ at 6 hours (Chu et al. 1992). The present study demonstrated that expression of IFN-γ mRNA was rare in any of the biopsy specimens, with no indication that PPD induced an upregulation in this cytokine expression at the 7 hour time point. This result would suggest that IFN-γ is not produced at high levels in the early ovine DTH and is not a major component in the control of the early cell migration, although previous work would suggest that the infiltrating cells are important producers of this cytokine. The depression in DTH size reported to occur following depletion of IFN-γ (Emery, Davey, 1995) would indicate a pivotal role for this cytokine in the development of the DTH response, although this work provides no indication of the kinetics of the involvement of IFN-γ in the reaction. A less probable

interpretation would be that the IFN-γ is being released from storage and functioning without the need for induction of mRNA production, although there is no evidence to support such a theory. A similar situation exists for the expression of mRNAs encoding for IL-2R, with this experiment indicating that expression rarely occurred in skin, and that PPD failed to induce an appreciable and consistent signal by 7 hours. Previous studies have reported the presence of considerable levels of IL-2, but only in biopsies of later time points (Tsicopoulos et al. 1992; Ohmen et al. 1995; Tsicopoulos et al. 1994; Ng et al. 1995), although one study reported the expression of IL-2 in untreated bovine skin (Ng et al. 1995). This would suggest that expression of IL-2, and hence the expression of it's receptor molecule, occurs later in the reaction and is probably associated with the presence of the T lymphocytes at this time point. It would be reasonable to assume that IFN-γ and IL-2R would be expressed simultaneously as they are both primary components of the Th1 type response.

The presence of TNF-α in PPD induced DTH responses has also been reported previously, although once again a majority of biopsies were taken at time points of 24 hours and later (Chu et al. 1992; Ng et al. 1995). Depletion of TNF-α has also been shown to reduce the DTH response (Chensue et al. 1994). In this study mRNA for TNF-α was seen at low levels in a significant proportion of the biopsies including untreated skin, although there was no consistent pattern of expression, as was reported previously in the bovine DTH (Ng et al. 1995). These findings correlate with a previous report that indicated the constitutive expression of TNF-α mRNA in untreated ovine skin at levels which remained unchanged when the skin was undergoing an inflammatory response (Elhay et al. 1994). Again, this would suggest that the expression of mRNAs for this cytokine is not specifically upregulated by the PPD at this early time point, although there is still a possibility that TNF-α may be playing a significant role at this time point as it has been shown to be stored in an active form in various cell types and released on stimulation without the requirement for the production of mRNAs (Vassalli, 1992). As a major proinflammatory cytokine

responsible for the induced migration of lymphocytes and PMNs (Colditz, Watson, 1992), it is probable that this cytokine plays a part in the DTH, but it would appear that the differential expression of this cytokine is not associated with the depression in DTH response associated with MVV infection. The phenomenon of the release of preformed TNF-α in the lesion would require a study using techniques such as monoclonal antibody staining that are not currently available.

The apparent high levels of IL-10 expression in a large number of the biopsies suggests that there is constitutive expression of this cytokine in the ovine skin. A previous study of the bovine DTH has indicated that IL-10 is expressed preferentially in DTH reactions compared to untreated skin (Ng et al. 1995), and IL-10 has been shown to occur in human tuberculin reactions (Ohmen et al. 1995). IL-10 is generally considered to be an immunosuppressive agent, and it's production by various cell types including keratinocytes has been recorded (Luger, Schwarz, 1995). Indeed, it has been postulated that IL-10 acts as a natural suppressant of cutaneous inflammation and acts to limit immunopathological damage (Berg et al. 1995), being produced in response to agents such as ultraviolet light (Luger, Schwarz, 1995). IL-10 is also involved in the switch between Th1 and Th2 subsets, and has been shown to inhibit DTH reactions (Powrie et al. 1993; Li et al. 1994). It is therefore not surprising that the mRNA encoding for this cytokine was found in a wide range of biopsies, where the expression is presumably involved in maintaining an immunological homeostasis in the skin. There was a suggestion that there may be a greater expression of IL-10 in the MVV infected sheep, although it should be stressed that the experiment was not performed in a manner that allows quantification of the results. Upregulation of IL-10 production has been previously reported in macrophages when they are infected with HIV or stimulated with HIV peptides *in vitro* (Akridge et al. 1994; Emilie et al. 1994; Haraguchi et al. 1995), and the operation of an analagous mechanism in MVV infected sheep could provide an explanation for the suppression of the DTH.

The lack of detectable viral RNA in the skin of the MVV infected sheep was a significant finding. This would provide evidence that the depression in early cell traffic in the DTH response is not as a direct result of the presence of viral particles or active virus transcription within the lesion, but must be occurring either as a result of the presence of latent viral DNA in the skin lesion or due to systemic immunosuppressive effects of viral infection.

In conclusion, this experiment has indicated that the depression in DTH associated with MVV infection is not associated with the local presence of virus RNA. There was no clear distinction in cytokine profiles between the MVV infected and control sheep, indicating that either the induction of these particular cytokines is not affected by the MVV infection, or that there is a quantitative rather than qualitative difference in cytokine production. Further studies could have included broadening the the range of inflammatory mediators studied, with one of the more promising candidates being the potent macrophage released proinflammatory agent interleukin-1. Broadening the time course of samples taken would also have been of great benefit as many mRNAs are only present during relatively short time periods, in particular the use of later time point biopsies during the maximal response would have been a logical progression.

CHAPTER TEN

FINAL DISCUSSION

The primary objective of this thesis was to study the *in vivo* functionality of the cell mediated immune system in sheep naturally infected with MVV, using the PPD induced dermal DTH reaction as an experimental model.

There was no previous report of a histological study the ovine PPD induced dermal DTH response, and the essential first stage of the experimental process was the characterisation of immunohistological characterisation of the DTH reaction in non-infected control sheep. The results of this experiment indicated that the gross DTH lesion consisted of a reddened indurative plaque that was maximal in size at 48-72 hours. Histologically, the response commenced at 7 hours post PPD injection, being characterised by the margination and perivascular emigration of PMNs. This was followed, at 24 hours, by the subsequent widespread emigration of large numbers of PMNs, particularly into the periadnexal area., with a less marked emigration of $CD4^+$ and $CD8^+$ lymphocytes. By 48 hours, the density of PMNs in the lesion had declined markedly, with an increase in the densities of both $CD4^+$ and $CD8^+$ lymphocytes. The subsequent 72 hour biopsy had similar characteristics, as did the 96 hour biopsy, although by this time the densities of inflammatory cells were lower. Fibrin and oedema were marked features of the post 7 hour reaction. Macrophage density remained at a relatively static level until 48 hours, at which time there was a marked reduction in cell numbers which remained low throughout the remainder of the reaction. The cell count of MHC class II expressing cells was similar to that of the macrophages, exhibiting similar kinetics, suggesting that these markers were staining the same cell population. Cell counts of B cells, mast cells and $\gamma\delta$ T cells remained relatively low throughout the reaction, suggesting that they played little part in the development of the lesion. These results constituted the basis of the further studies in

the thesis, and also has been published (Pyrah & Watt, 1995). In particular, the finding of a significant presence of PMNs in the early lesion clarifies the conflicting reports in previous publications. As such, the work has relevance to the general understanding of the cellular basis of the DTH response in all species.

The next experiment investigated the effect of the presence of natural MVV infection on the development of a DTH response, and attempted to relate the findings with the degree of classical maedi lung pathology. There were no previous studies of the DTH response in naturally infected sheep, although a similar investigation in experimentally infected sheep had produced some evidence for the depression of DTH in experimentally infected sheep (Myer et al. 1988). The results presented here indicate that natural MVV infection was associated with a significant and variable depression in the increase in skin thickness in the DTH response, and that this depression was not associated with the degree of pathological change in the animal. The former finding is consistent with the results of analogous studies in HIV infection, although the latter finding is contrary to HIV research that linked the depression in DTH with the progression of infection, in particular the development of AIDS.

The preceeding study provided the platform for the immunohistological assessment of the DTH response in MVV infected sheep. This showed that the depression in DTH response in MVV infected was associated with a reduction in the density of PMNs and $CD4^+$ cells in the reaction at 24 hours, and that the density of cells in the later lesion was not altered. These results suggested that the PMN and $CD4^+$ cell were essential for the production of the induration, and that MVV infection resulted in the reduced ability of these cells to enter a DTH lesion. Although there were many reports of the decline in DTH response in HIV infected individuals, there were no reports of similar investigations into the histological basis of the alteration in DTH development. This experiment therefore contributed to a greater understanding

of the defect in DTH response in MVV infected sheep and also had comparative interest in the study of the pathogenesis of HIV infection.

As no specific evidence that the PMN was involved in the development of the DTH reaction was available, control sheep were subsequently depleted of circulating PMNs using hydroxyurea. Although this treatment was not successful in all cases, there was an association between the circulating number of PMNs and the development of a DTH reaction and degree of $CD4^+$ cell influx into the lesion, indicating that the PMN played a significant role in the reaction. These findings are of significance to the study of alterations in the DTH reaction and may contribute to understanding of the mode of action of the antigen specific cell mediated immune response.

Once the importance of the PMN in the DTH had been established, it was logical to study the *in vivo* performance of this cell and the $CD4^+$ cell in the MVV infected sheep, in order to ascertain whether the reduction in PMN and $CD4^+$ cell density in the early DTH lesion was as a result of dysfunction of these particular cells in the sheep, or whether they were responding normally to an aberrant inflammatory stimulus. To investigate this phenomenon, a necessarily limited range of proinflammatory agents that were known to attract significant levels of PMNs and $CD4^+$ cells were injected intradermally into MVV infected and matched control sheep. These agents acted via distinct mechanisms, but there was no significant difference in the level of PMN and $CD4^+$ cell emigration stimulated, and no correlation between the emigration and the degree of depression of a subsequent DTH response. This provided evidence that the DTH defect occurred as a result of an alteration in the balance of inflammatory signal in the early part of the DTH response, and is the first reported study of the *in vivo* migratory abilities of inflammatory cells in sheep infected with MVV.

These results indicated that the defect in DTH development in the MVV infected sheep was associated with an alteration in the proinflammatory signal produced by the early DTH lesion, and which was responsible for the emigration of primarily PMNs. The fact that the PMN migration occurred at high levels in the early lesion suggested that antibody/antigen interaction and complement activation may have been significantly involved in the DTH immune response. A study of circulating anti-PPD antibodies was therefore undertaken in order to establish whether there was any difference in the response to BCG vaccination in the MVV infected sheep. This experiment indicated that there was an insignificant antibody response to BCG vaccination when compared to hyperimmune serum, confirming that there was a predominant stimulation of the cell mediated immune system. There was, however, also limited evidence that there were higher titres of antibodies in MVV infected sheep, with a negative correlation between antibody titre and increase in skin thickness of the DTH response. This finding suggested that there might be an MVV associated shift in immune response from a Th1 to a Th2 type response, analogous to the situation reported in HIV infection.

The indication that antibodies were not involved in the promotion of PMN migration lead to the investigation of other inflammatory mediators in the developing DTH. As the lesion was relatively small at the 7 hour time point at which cell migration had begun, the highly sensitive and highly specific technique of RT-PCR followed by internal radiolabelled oligonucleotide probing was utilised to investigate the mRNA production of a range of cytokines. The cytokines chosen for study were either reported initiators of the DTH response, or had been shown to specifically depress the DTH response *in vivo*. This study was the first reported investigation of the cytokine profile of the developing tuberculin type DTH response, and one of the few records of cytokine production in the normal skin of the sheep. The study indicated that there was constitutive expression of TNF-α and IL10 in normal untreated skin, although there was no specific upregulation of IL2R, IL10, TNF-α,or

IFN-γ at 7 hours post injection of either PBS or PPD. There was some evidence for a trend towards higher levels of IL10 expression in MVV infected animals. Most surprisingly, there was no viral RNA present in any of the skin biopsies examined. This latter finding indicates that the depression of DTH response is either associated with a systemic effect of the virus, or that the effect is resultant from the presence of viral DNA in an inactive or latent form. The author is not aware of any similar study of virus presence in the DTH lesion of HIV infected patients.

In conclusion, this thesis has contributed to the understanding of the normal DTH response and has indicated the immunopathological mechanism by which this reaction is depressed in sheep naturally infected with MVV.

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APPENDICES

APPENDICES TO CHAPTER 2

Appendix 2.9.2

Red blood cell lysis buffer solutions (freshly prepared immediately prior to use):

90mls	0.16M NH_4CL_3
10mls	017M TRIS (pH 7.65)

The resultant solution adjusted to pH 7.2.

Cell washing buffer

Phosphate Buffered Saline with:

1%	Bovine Serum Albumin
0.5%	Heparin
0.1%	Sodium Azide

Appendix 2.11 3

ELISA alkaline phosphatase substrate

Per 10 mls (sufficient to do one plate)

5ml	0.2M glycine
2.7ml	0.2M NaOH
5µl	1M $MgCl_2$
50µl	0.1M $ZnCl_2$
2.2ml	Dist. H_2O
10mg	Sigma powder 104 (Sigma-Aldrich Company Ltd., Dorset, England)

Appendix2.11.4

SDS PAGE gel solutions

	Stacking gel	**5% gel**	**20% gel**
30% acrylamide / 0.8% bis	0.5ml	2ml	8.3ml
1M tris / 5mM EDTA pH 8.8		3.8ml	3.8ml
Distilled water	2.5ml	6.25ml	
10% SDS	50µl	125µl	125µl
1M tris pH 6.8	0.6ml		
**10% Ammonium persulphate(freshly made up)*	25µl	30µl	30µl
**Temed*	7.5µl	3µl	3µl

**ONLY ADDED IMMEDIATELY PRIOR TO POURING THE GEL*

SDS dissolving buffer

25 mM tris pH 7.5	250µl 1M tris
2% SDS	2ml 10% SDS
20% glycerol	2ml glycerol
0.01% bromophenol blue	1mg
5% 2-mercaptoethanol	0.5ml
water	5.25ml

10x running buffer for SDS-PAGE

Tris	30g
Glycine	144g
EDTA (di-sodium salt)	7.44g
SDS	10g

Make up to 1 litre with distilled water

Tris/glycine/methanol western blotting buffer

Tris	11.43g
Glycine	5.85g
Methanol	400ml

Make up to 2 litres in water

Appendix 2.13.1

Reverse transription mix

x5 RT superscript buffer (Gibco BRL)	5µl
Random hexamer primers (Pharmacia Biotech)	1.5µl
20nm dNTPs (Pharmacia Biotech)	2.5µl
0.1mDTT (Gibco BRL)	1µl
RNAsin (Pharmacia Biotech)	1µl
RT superscript (Gibco BRL)	1µl
RNase free water (Sigma)	3µl

Appendix 2.13.2

PCR reaction mix

PCR buffer [100mM Tris HCl,pH 8.3,500mM KCl] (Gibco BRL)	5.0 µl
50mM $MgCl_2$ (Gibco BRL)	2.5 µl
20mM dNTPs (Pharmacia Biotech)	1.0 µl
Primer 1 (25pmol/µl)	1.0 µl
Primer 2 (25pmol/µl)	1.0 µl
cDNA	3.0 µl
RNA water (Sigma)	36.0 µl

Appendix 2.13.3

TAE buffer (0.04M tris-acetate, 0.001M EDTA)

242g	tris base
57.1ml	glacial acetic acid
100ml	0.5M EDTA (pH 8.0)

Make up to one litre with distilled water.

Appendix 2.14.1

SSPE buffer (x20 concentrate)

175.3g NaCl 27.6g $NaH_2PO_4.H_20$ (Equivalent to 31.2g of $NaH_2PO_4.2H_20$)
7.4g EDTA

Make up to 800ml with water.

pH to 7.0 with 10N NaOH.

Adjust the volume to 1000ml with distilled water and sterilise by autoclaving

Appendix 2.14.2

Hybridisation buffer (per 10 mls)

8.46ml distilled H_2O
1g dextran sulphate
1ml 10% SDS

Incubate for 30 minutes at 65°C, and then add

0.58g NaCl
0.4ml 20xSSPE

Incubate for 15 minutes at 65°C prior to use

TE Buffer

10mM TRIS-HCl (pH 7.4)
1mM EDTA (pH 8.0)

Radiolabelling mix

2.0µl	10x PNK buffer
1.0µl	oligonucleotide (at a concentration of 50ng/µl)
14.0µl	RNAase free water
2.0µl	^{32}P labelled ATP (equivalent to 20µCi)
1.0µl	PNK enzyme

Incubate for 30 minutes at 37°C, and then terminate the reaction with

80.0µl	TE buffer

APPENDICES TO CHAPTER 3

Appendix 3.3.1

Table of increase in skin thickness versus time post PPD injection

Sheep number	Increase in skin thickness (mm) at					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	0	0	1	6	8.5	6
114	0	0	3	16	14	6
142	0	0	4	14	16	8
143	0	1	6	14	14	8
144	0	0	5	9	13	8
145	0	0	4	12	14	8

Table of increase in skin thickness versus time post control (PBS) injection

Sheep number	Increase in skin thickness (mm) at					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	0	0	0	0	0	0
114	0	0	0	0	0	0
142	0	0	0	0	0	0
143	0	0	0	0	0	0
144	0	0	0	0	0	0
145	0	0	0	0	0	0

Appendix 3.3.3

Tables indicating the superficial dermal and periadnexal cell count of PMN cells at time points following the i/d injection of PPD and PBS in sensitised sheep

Sheep number	Superficial dermal PMN cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	1	1	1	0	1	9
114	8	21	16	2	0	2
142	4	2	3	0	2	0
143	2	8	4	9	8	8
144	6	4	2	8	2	2
145	1	8	0	0	6	0
	Superficial dermal PMN cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	59	129	51	112	93	46
114	170	120	154	89	49	8
142	12	2	139	51	67	28
143	5	2	142	157	43	18
144	3	4	132	42	42	7
145	0	12	196	0	100	16

Sheep number	Periadnexal PMN cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	1	5	7	2	7	7
114	13	37	19	6	8	11
142	10	4	5	2	8	5
143	4	10	26	9	1	4
144	5	6	5	8	8	6
145	3	2	1	4	8	6
	Periadnexal PMN cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	82	140	42	104	56	35
114	177	116	236	79	93	24
142	10	12	199	53	85	69
143	10	8	209	158	74	36
144	6	13	205	64	45	22
145	2	27	216	8	128	36

Appendix 3.3.4

Tables indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SBU-T4 ($CD4^+$ T cells) at time points following the i/d injection of PPD and PBS in sensitised sheep

Sheep number	Superficial dermal CD4 cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	4	6	4	1	4	4
114	6	1	39	9	2	4
142	4	0	1	3	4	8
143	2	4	4	4	8	8
144	12	4	10	17	8	4
145	6	1	1	4	2	4
	Superficial dermal CD4 cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	6	10	20	52	88	68
114	10	5	8	98	112	72
142	10	4	30	57	102	60
143	10	2	39	64	65	138
144	12	5	19	46	77	74
145	8	8	16	54	64	62

Sheep number	Periadnexal CD4 cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	5	16	17	20	19	16
114	9	16	58	45	21	8
142	6	3	0	4	10	17
143	4	8	8	4	9	10
144	9	4	7	28	9	14
145	10	6	5	8	12	9
	Periadnexal CD4 cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	3	56	21	158	205	197
114	27	17	29	181	160	116
142	7	5	34	90	121	172
143	3	9	69	147	152	126
144	12	16	44	83	108	88
145	22	10	50	110	120	106

Appendix 3.3.5

Tables indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SBU-T8 ($CD8^+$ T cells) at time points following the i/d injection of PPD and PBS in sensitised sheep

Sheep number	Superficial dermal CD8 cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	3	5	7	7	4	1
114	5	5	8	5	6	5
142	4	7	5	0	1	2
143	0	3	1	3	1	0
144	6	4	6	8	6	4
145	4	1	0	1	1	1
	Superficial dermal CD8 cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	5	4	6	49	38	45
114	1	6	29	85	94	76
142	2	2	1	18	22	29
143	3	1	8	17	31	22
144	5	4	33	24	26	19
145	0	3	6	7	11	7

Sheep number	Periadnexal CD8 cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	4	7	17	5	3	6
114	9	8	19	9	16	12
142	2	8	6	4	1	3
143	1	2	1	4	2	2
144	5	2	9	5	1	2
145	4	3	2	2	3	2
	Periadnexal CD8 cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	7	7	25	124	112	109
114	12	19	39	187	155	114
142	4	1	4	55	54	69
143	7	4	12	51	33	45
144	6	6	58	50	48	40
145	3	3	9	47	24	34

Appendix 3.3.6

Tables indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody 86D (gamma delta T cells) at time points following the i/d injection of PPD and PBS in sensitised sheep

Sheep number	Superficial dermal g/d cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	1	11	7	3	2	9
114	4	6	20	7	5	5
142	0	6	14	12	14	5
143	1	1	2	3	4	3
144	9	1	0	13	0	2
145	3	1	2	3	3	2
	Superficial dermal g/d cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	5	2	2	15	8	16
114	5	7	14	37	32	13
142	1	1	5	1	5	7
143	1	1	4	2	2	4
144	0	7	9	9	18	6
145	5	1	4	8	12	6

Sheep number	Periadnexal g/d cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	4	16	15	7	10	8
114	14	10	33	11	15	5
142	4	5	5	10	8	4
143	3	2	4	8	4	4
144	13	2	1	3	1	4
145	6	5	6	5	3	6
	Periadnexal g/d cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	6	5	11	19	24	33
114	23	27	29	31	39	29
142	1	3	2	3	6	8
143	0	2	8	6	7	8
144	1	6	8	6	12	7
145	7	3	3	8	10	11

Appendix 3.3.7

Tables indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody OM1 (macrophages) at time points following the i/d injection of PPD and PBS in sensitised sheep

Sheep number	Superficial dermal OM1 cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	5	8	7	6	8	7
114	4	7	5	8	6	5
142	3	4	5	7	5	3
143	3	2	13	21	2	2
144	2	3	2	1	5	4
145	2	10	3	3	3	5
	Superficial dermal OM1 cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	5	6	3	4	2	7
114	3	5	2	1	0	2
142	4	5	2	0	0	0
143	5	4	1	0	1	0
144	4	8	0	1	0	0
145	10	8	4	8	1	1

Sheep number	Periadnexal OM1 cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	12	13	14	12	5	7
114	10	11	15	12	9	12
142	5	16	12	17	15	14
143	7	3	7	23	12	1
144	10	10	8	6	7	9
145	23	25	19	13	8	5
	Periadnexal OM1 cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	10	12	13	5	6	10
114	9	15	4	3	2	2
142	9	12	4	3	1	1
143	13	21	3	2	1	2
144	13	16	1	0	1	2
145	20	10	12	8	2	0

Appendix 3.3.8

Tables indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SW73.2 (MHC class II) at time points following the i/d injection of PPD and PBS in sensitised sheep

Sheep number	Superficial dermal SW73.2 cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	5	4	3	4	2	2
114	4	4	4	3	3	5
142	3	3	5	2	4	4
143	2	6	4	2	4	5
144	6	3	3	3	3	3
145	2	4	2	3	4	3
	Superficial dermal SW73.2 cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	5	3	2	0	0	0
114	6	2	1	0	0	0
142	2	3	3	1	0	0
143	2	4	1	0	3	0
144	2	5	2	2	0	0
145	2	4	0	0	1	0

Sheep number	Periadnexal SW73.2 cell count following PBS injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	8	7	6	8	7	9
114	10	12	10	8	7	8
142	13	11	10	9	8	9
143	10	13	15	5	12	15
144	11	9	10	11	8	9
145	9	11	9	8	11	9
	Periadnexal SW73.2 cell count following PPD injection					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
111	9	8	0	1	0	0
114	12	14	2	0	0	0
142	11	9	5	0	0	0
143	9	2	0	0	1	0
144	12	2	0	1	0	0
145	12	0	1	2	2	1

APPENDICES TO CHAPTER 4

Appendix 4.3.1

Table indicating the increase in skin thickness at time points following the i/d injection of PPD in sensitised sheep

Sheep number	**Increase in skin thickness (mm) at**					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
004	0	0	3	5	7	6
10	0	0	0	2	2	2
20	0	0	0	3	2	2
27	0	0	0	16	10	7
57	0	0	0	4	5	3

Table indicating the increase in skin thickness at time points following the i/d injection of PBS in sensitised sheep

Sheep number	**Increase in skin thickness (mm) at**					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
004	0	0	0	0	0	0
10	0	0	0	0	0	0
20	0	0	0	0	0	0
27	0	0	0	0	0	0
57	0	0	0	0	0	0

Appendix 4.3.2

Results of the assessment of the three parameters of lung pathology by two independent pathologists

sheep number	status	interstitial score		smooth muscle score		number of follicles	
		scorer 1	scorer 2	scorer 1	scorer 2	scorer 1	scorer 2
4	mvv +ve	5	5	4	4	66	80
10	mvv +ve	1	0	4	0	0	0
20	mvv +ve	3	0.5	9	1.5	0	0
27	mvv +ve	15	11	15	9	48	81
57	mvv +ve	1	0	4	0	0	0
111	mvv -ve	3	1.5	11	3.5	0	0
114	mvv -ve	2	2.5	10	3	1	1
142	mvv -ve	4	3.5	9	3.5	0	1
143	mvv -ve	0	0	5	0	0	0
144	mvv -ve	3	0.5	8	1.5	0	0
145	mvv -ve	1	0	5	0	0	0

Appendix 4.3.4

Table indicating the value of the Spearman correlation statistic relating the lung pathology assessment attributed independently by two pathologists and the increase in skin thickness at time points following i/d PPD injection in sensitised control and MVV infected sheep. There are no significant correlations.

	Increase in skin thickness at					
	2 hours	**7 hours**	**24 hours**	**48 hours**	**72 hours**	**96 hours**
Interstitial score 1	0	-0.509	-0.195	0.195	-0.019	0.048
Interstitial score 2	0	-0.359	-0.095	0.447	0.179	0.104
Smooth muscle hyperplasia score 1	0	-0.152	-0.064	0.597	0.324	0.191
Smooth muscle hyperplasia score 2	0	-0.360	-0.127	0.380	0.113	0.082
Follicle structure count 1	0	-0.191	-0.164	0.372	0.006	-0.066
Follicle structure count 2	0	-0.191	0.218	0.256	0.317	0.072

APPENDICES TO CHAPTER 5

Appendix 5.2.1

Table of serological test results for the sheep used in this experiment

Sheep number	**Result of serological AGIDT test**									
	17/8/90	**20/11/90**	**19/12/90**	**25/1/91**	**18/6/91**	**17/6/92**	**20/4/93**	**10/2/94**	**20/6/94**	**2/11/94**
011 INF	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	ND	+ve
025 INF	+ve	+ve	+ve	+ve	ND	+ve	+ve	+ve	ND	+ve
035 INF	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	ND	ND
071 INF	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	ND	+ve
076 INF	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	ND	ND
118 INF	+ve	+ve	+ve	+ve	ND	+ve	+ve	+ve	ND	+ve
183 CON	ND	ND	ND	ND	ND	ND	ND	-ve	-ve	-ve
184 CON	ND	ND	ND	ND	ND	ND	ND	-ve	-ve	-ve
245 CON	ND	ND	ND	ND	ND	ND	ND	ND	-ve	-ve
246 CON	ND	ND	ND	ND	ND	ND	ND	ND	-ve	-ve
247 CON	ND	ND	ND	ND	ND	ND	ND	ND	-ve	-ve

Appendix 5.2.4

Computer image analysis routine (QUIPS) routine used for cell counting

```
Expression  ( TAG=1 )
For  ( TAG = 1 to 6, step 1 )
  PauseText  ( "Draw around the gland and then press continue or O.K." )
  Binary Edit  [PAUSE]  ( Draw from Detected to Detected, nib Fill, width 2 )
  Binary Amend  ( Dilate from Detected to Amended, cycles 100 )
  Binary Logical  ( C = A XOR B :  C Amended, A Detected, B Amended )
  Expression  ( MFLDIMAGE=3 )
  Measure field  ( plane MFLDIMAGE, into FLDRESULTS(1) )
     Selected parameters:     Area
  Binary Logical  ( copy Amended, inverted to Amended  )
  Binary Logical  ( copy Amended to Accepted  )
  PauseText  ( "Indicate the positive cells using the mouse and then press OK or
continue" )
  Binary Edit  [PAUSE]  ( Line from Amended to Amended, nib Circle, width 6 )
  Binary Logical  ( C = A XOR B :  C Accepted, A Amended, B Accepted )
  Expression  ( MINAREA=0 )
  Expression  ( MFEATINPUT=4 )
  Expression  ( FERETS=8 )
  Measure feature  ( plane MFEATINPUT, FERETS ferets, minimum area:
MINAREA
        feature counts into FTRCOUNT(2), results into FTRRESULTS(count,0) )
     Selected parameters:
  Display Page
  Display  ( "Area measured around the gland (mmsquare)" )
  Display  ( FLDRESULTS(1) )
  Display Line
  Display  ( "Cells counted" )
  Expression  ( CELLCOUNT=FTRCOUNT(1) )
  Display  ( CELLCOUNT )
  Display Line
  Display  ( "Cells per area (cells/mm2)" )
  Expression  ( AREAPERCELL=FTRCOUNT(1)/FLDRESULTS(1) )
  Display  ( AREAPERCELL )
  Binary Logical  ( C = A XOR B :  C Detected, A Detected, B Detected )
  Binary Logical  ( copy Detected to Amended  )
  Binary Logical  ( copy Detected to Accepted  )
Next  ( TAG )
Expression  ( TAG2=1 )
For  ( TAG2 = 1 to 6, step 1 )
  PauseText  ( "Draw a superficial dermal area ignoring adnexae and lymphatics" )
```

```
  Binary Edit  [PAUSE]  ( Draw from Detected to Detected, nib Fill, width 2 )
  Expression   ( MFLDIMAGE=2 )
  Measure field   ( plane MFLDIMAGE, into FLDRESULTS(1) )
     Selected parameters:     Area
  Binary Logical   ( copy Detected, inverted to Detected  )
  Binary Logical   ( copy Detected to Amended  )
  PauseText   ( "Indicate the positive cells with the mouse" )
  Binary Edit  [PAUSE]  ( Line from Detected to Detected, nib Circle, width 6 )
  Binary Logical   ( C = A XOR B :  C Amended, A Detected, B Amended )
  Expression   ( MFEATINPUT=3 )
  Expression   ( MINAREA=0 )
  Expression   ( FERETS=8 )
  Measure feature   ( plane MFEATINPUT, FERETS ferets, minimum area:
MINAREA
        feature counts into FTRCOUNT(2), results into FTRRESULTS(count,0) )
     Selected parameters:
  Display Page
  Display   ( "Area measured (mm2)" )
  Display   ( FLDRESULTS(1) )
  Display Line
  Display   ( "Cells counted" )
  Display   ( FTRCOUNT(1) )
  Display Line
  Display   ( "Cells per mm2" )
  Display   ( FTRCOUNT(1)/FLDRESULTS(1) )
  Binary Logical   ( C = A XOR B :  C Detected, A Detected, B Detected )
  Binary Logical   ( copy Detected to Amended  )
 Next   ( TAG2 )
```

Appendix 5.3.1

Table indicating the increase in skin thickness at time points following the i/d injection of PPD in sensitised MVV positive and control sheep

Sheep number	**Increase in skin thickness (mm) at**					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
011	0	0	4	8	7	5.5
025	0	2	5.33	6.5	5.5	2
035	0	0	2.667	4.5	5.5	4
071	0	0.333	2.667	4.5	6	2
076	0	0	1	1	3	2
118	0	0	3.333	8	8.5	2
183	0	1.333	6.667	11.5	10	5
184	0	0.667	5	11.5	10	5
245	0	1.5	10.666	14.5	13	7
246	0	1	10.666	15	11.5	5
247	0	1	8.333	16	13	7

Table indicating the increase in skin thickness at time points following the i/d injection of PBS in sensitised sheep

Sheep number	**Increase in skin thickness (mm) at**					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
011	0	0	0	0	0	0
025	0	0	0	0	0	0
035	0	0	0	0	0	0
071	0	0	0	0	0	0
076	0	0	0	0	0	0
118	0	0	0	0	0	0
183	0	0	0	0	0	0
184	0	0	0	0	0	0
245	0	0	0	0	0	0
246	0	0	0	0	0	0
247	0	0	0	0	0	0

Appendix 5.3.3

Table indicating the superficial dermal and periadnexal cell count of PMN cells at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	177	192	188	105	100	85
25	158	194	194	139	113	139
35	188	213	221	78	98	127
71	315	291	177	226	170	134
76	207	186	169	105	102	99
118	215	225	204	78	127	84
183	164	108	187	77	71	91
184	184	183	190	102	100	101
245	184	119	117	74	96	99
246	122	117	154	81	71	74
247	180	96	140	80	72	69

Table indicating the superficial dermal and periadnexal cell count of PMN cells at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	177	2526	696	105	1589	309
25	158	2628	1114	139	1201	274
35	188	3935	671	78	2395	492
71	315	1226	1245	226	1120	778
76	207	979	1317	105	713	577
118	215	2364	1519	78	2030	1183
183	164	5094	2756	77	2841	1877
184	184	4125	2822	102	2621	1942
245	184	5271	1425	74	2513	887
246	122	5111	2118	81	1768	1673
247	180	4561	732	80	1937	490

Appendix 5.3.4

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SBU-T4 ($CD4^+$ T cells) at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	239	187	248	105	112	141
25	309	249	270	148	123	141
35	381	187	263	141	124	118
71	174	223	294	133	106	129
76	245	322	183	139	128	92
118	286	215	234	129	123	134
183	310	201	272	142	125	132
184	247	178	183	111	104	121
245	219	251	240	123	156	157
246	227	229	298	126	121	169
247	238	214	263	138	128	132

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SBU-T4 ($CD4^+$ T cells) at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	239	750	1893	105	500	1078
25	309	437	2325	148	362	1625
35	381	1008	2076	141	306	1131
71	174	750	1893	133	500	1078
76	245	859	2303	139	567	1286
118	286	851	1878	129	427	699
183	310	1179	2082	142	740	1091
184	247	1217	2367	111	556	1212
245	219	979	1763	123	601	1084
246	227	1024	1872	126	478	1077
247	238	1305	1834	138	614	1027

Appendix 5.3.5

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SBU-T8 ($CD8^+$ T cells) at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	248	199	275	102	103	143
25	177	215	160	134	126	99
35	232	204	182	115	121	126
71	156	177	197	113	112	100
76	176	207	204	120	101	116
118	203	185	188	116	124	135
183	166	154	174	109	114	112
184	227	224	239	127	132	160
245	96	171	302	100	117	109
246	178	224	217	108	137	108
247	225	250	232	133	147	160

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SBU-T8 ($CD8^+$ T cells) at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	248	260	934	102	182	521
25	177	505	1008	134	281	582
35	232	433	1285	115	201	467
71	156	507	1026	113	291	566
76	176	523	1301	120	292	403
118	203	431	1365	116	213	497
183	166	416	1208	109	256	446
184	227	482	1214	127	305	581
245	96	654	1003	100	363	652
246	178	601	1172	108	354	665
247	225	709	1152	133	430	622

Appendix 5.3.6

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody 86D (gamma delta T cells) at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	109	246	184	53	93	88
25	130	460	357	143	172	171
35	245	302	148	67	161	100
71	122	272	227	93	151	139
76	184	265	114	124	117	93
118	213	216	138	79	190	101
183	139	201	206	97	158	120
184	152	102	90	97	57	51
245	150	131	153	87	116	86
246	146	162	68	109	108	89
247	108	170	150	71	86	64

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody 86D (gamma delta T cells) at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	109	114	158	53	114	120
25	130	273	187	143	293	215
35	245	138	153	67	80	132
71	122	271	261	93	110	135
76	184	229	457	124	135	94
118	213	221	139	79	120	106
183	139	307	185	97	120	137
184	152	159	173	97	93	137
245	150	192	143	87	144	123
246	146	412	318	109	177	193
247	108	354	341	71	236	139

Appendix 5.3.7

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody OM1 (macrophages) at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	74	82	88	144	162	126
25	124	112	128	173	175	231
35	181	125	184	140	174	190
71	117	127	161	136	151	127
76	93	155	148	143	172	140
118	65	116	84	94	120	122
183	140	129	178	151	127	167
184	117	143	136	133	163	139
245	125	92	101	106	105	116
246	221	158	161	152	132	114
247	209	169	147	130	242	142

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody OM1 (macrophages) at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	74	43	45	144	95	87
25	124	18	13	173	73	65
35	181	70	14	140	138	37
71	117	16	13	136	38	35
76	93	27	101	143	63	125
118	65	19	12	94	20	23
183	140	32	36	151	21	125
184	117	37	21	133	89	50
245	125	117	10	106	132	97
246	221	129	10	152	108	29
247	209	144	10	130	185	50

Appendix 5.3.8

Tables indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SW73.2 (MHC class II) at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	557	475	506	450	424	314
25	517	490	483	321	275	298
35	368	429	354	249	227	256
71	596	539	324	416	295	287
76	416	420	398	249	218	273
118	441	420	379	248	266	251
183	367	312	442	234	245	265
184	356	356	353	286	256	237
245	526	518	533	351	381	320
246	611	431	400	354	315	370
247	477	490	383	234	350	241

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SW73.2 (MHC class II) at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
11	557	987	100	450	375	209
25	517	515	100	321	468	380
35	368	633	100	249	370	232
71	596	659	100	416	400	250
76	416	536	100	249	560	240
118	441	614	100	248	430	286
183	367	852	50	234	550	242
184	356	713	50	286	457	239
245	526	590	50	351	673	341
246	611	721	50	354	274	328
247	477	663	50	234	486	214

APPENDICES TO CHAPTER 6

Appendix 6.3.1

Table indicating the total white blood cell count (cells x 10^9 l^{-1}) during the course of hydroxyurea treatment (25/7/95 - 30/7/95) and subsequent DTH response, with the PPD injected intradermally on 31/7/95

Sheep no	25/07/95	26/07/95	27/07/95	28/07/95	29/07/95	30/07/95	31/07/95	01/08/95	02/08/95	03/08/95
33	6.5	7.8	7.1	5.9	5.9	4.5	4.8	3.2	3.7	4.7
34	7.1	8.7	8.8	7.9	8.1	5	4.7	5.1	6	6.3
35	7.3	7.1	6.7	7.4	15.2	12.6	10.3	13.9	12.3	15.6
36	27	25.8	23	22	17.7	20.6	20.2	14.1	15	12.6
37	5.8	5.7	6.4	11.2	4.6	3.4	3.1	3.9	10.5	4.7
38	8.4	10.3	9	5.7	16.8	15.5	13.1	13.1	4.3	13.7

Table indicating the segmented PMN cell count (cells x 10^9 l^{-1}) during the course of hydroxyurea treatment (25/7/95 - 30/7/95) and subsequent DTH response, with the PPD injected intradermally on 31/7/95

Sheep no	25/07/95	26/07/95	27/07/95	28/07/95	29/07/95	30/07/95	31/07/95	01/08/95	02/08/95	03/08/95
33	1.69	1.482	1.775	1.298	1.18	0.675	0.24	0.48	0.518	0.517
34	2.201	2.61	3.256	1.975	2.511	0.85	1.14	1.275	0.6	1.134
35	2.92	2.414	1.474	1.702	3.95	3.15	1.957	7.228	4.92	3.432
36	13.77	11.352	8.28	8.14	5.31	8.858	9.292	8.178	6.3	2.52
37	1.508	2.451	1.984	4.48	1.15	0.136	0.279	0.819	2.205	1.833
38	2.856	2.781	2.97	1.254	6.216	4.65	3.668	6.681	1.591	2.74

Table indicating the lymphocyte cell count (cells x 10^9 l^{-1}) during the course of hydroxyurea treatment (25/7/95 - 30/7/95) and subsequent DTH response, with the PPD injected intradermally on 31/7/95

Sheep no	25/07/95	26/07/95	27/07/95	28/07/95	29/07/95	30/07/95	31/07/95	01/08/95	02/08/95	03/08/95
33	4.095	5.496	4.402	4.012	4.189	3.42	4.32	2.656	2.849	3.76
34	4.189	4.959	4.4	4.898	5.022	4	2.961	3.468	4.92	4.599
35	3.723	4.331	4.489	4.81	10.85	8.316	7.725	6.394	6.396	11.076
36	7.56	8.256	9.89	8.8	8.85	8.446	7.878	5.922	7.8	7.56
37	3.422	2.85	3.84	5.824	3.128	2.992	2.604	2.847	7.56	2.773
38	4.956	9.798	5.49	4.332	10.08	10.385	9.432	6.288	2.408	10.686

Table indicating the eosinophil cell count (cells x 10^9 l^{-1}) during the course of hydroxyurea treatment (25/7/95 - 30/7/95) and subsequent DTH response, with the PPD injected intradermally on 31/7/95

Sheep no	25/07/95	26/07/95	27/07/95	28/07/95	29/07/95	30/07/95	31/07/95	01/08/95	02/08/95	03/08/95
33	0.715	0.546	0.852	0.531	0.354	0.27	0.144	0	0.074	0.141
34	0.355	0.783	0.44	0.869	0.243	0.1	0.235	0.357	0.3	0.315
35	0.365	0.284	0.536	0.74	1.368	0.191	0.618	0	0.492	0.312
36	4.86	4.902	4.14	0	3.363	2.678	2.424	0	0.9	2.142
37	0.464	0.228	0.448	0.784	0.184	0.136	0.186	0	0.315	0.047
38	0.084	0.103	0.09	0.057	0.336	0.155	0	0	0.129	0

Table indicating the monocyte cell count (cells x 10^9 l^{-1}) during the course of hydroxyurea treatment (25/7/95 - 30/7/95) and subsequent DTH response, with the PPD injected intradermally on 31/7/95

Sheep no	25/07/95	26/07/95	27/07/95	28/07/95	29/07/95	30/07/95	31/07/95	01/08/95	02/08/95	03/08/95
33	0.065	0.234	0.071	0	0.118	0.09	0.096	0.064	0.185	0.235
34	0.213	0.087	0.44	0.079	0.243	0	0.094	0	0.06	0.252
35	0.292	0	0.201	0	0.304	0.252	0	0.278	0.492	0.78
36	0.81	1.29	0.46	4.84	0.177	0.412	0.202	0	0	0.378
37	0.406	0.171	0.128	0.0112	0.138	0.068	0.031	0.234	0.21	0.047
38	0.252	0.412	0.45	0.057	0.336	0.155	0	0.131	0.172	0.274

Table indicating the percentages of $CD4^+$ and $CD8^+$ cells prior to and following treatment with hydroxyurea, with the percentages of lymphocytes flourescing in the control tubes also indicated.

	% of lymphocytes fluorescing									
	No antibody		NMS		VPM53		CD4		CD8	
Sheep no	pre	post	pre	post	pre	post	pre	post	pre	post
33	2.98	1.2	0.67	1.42	0.65	1.34	29.11	36.14	28.36	31.87
34	0.67	1.4	0.79	2.08	0.96	1.45	18.14	20.99	21.44	19.78
35	1.65	2.22	2.84	1.77	1.84	1.72	21.64	26.46	35.29	30.03
36	4.63	1.43	2.96	1.78	2.77	1.3	26.52	33.37	29.16	25.76
37	1.46	2.1	2.16	2.28	1.37	3.88	28.17	31.71	33.23	26.07
38	0.86	1.3	1.69	1.58	0.94	1.09	22.42	17.99	51.53	44.21

Appendix 6.3.2

Table indicating the increase in skin thickness at time points following the i/d injection of PPD in sensitised PMN depleted and control sheep

Sheep number	Increase in skin thickness (mm) at					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
33	ND	ND	2.7	5.5	7	6
34	ND	ND	3.7	5	5	4.5
35	ND	ND	3.8	6.5	9.5	9
36	ND	ND	2.7	6	7.5	7
37	ND	ND	4.7	4.5	6	5
38	ND	ND	2.7	7	9	10
183	0	1.333	6.7	11.5	10	5
184	0	0.667	5	11.5	10	5
245	0	1.5	10.7	14.5	13	7
246	0	1	10.7	15	11.5	5
247	0	1	8.3	16	13	7

Appendix 6.3.4

Table indicating the superficial dermal and periadnexal cell count of PMN cells at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	211	200	164	81	152	86
34	148	176	141	98	113	74
35	149	178	255	118	85	70
36	180	165	183	68	67	73
37	156	335	330	65	134	120
38	307	225	166	145	68	101
183	164	108	187	77	71	91
184	184	183	190	102	100	101
245	184	119	117	74	96	99
246	122	117	154	81	71	74
247	180	96	140	80	72	69

Table indicating the superficial dermal and periadnexal cell count of PMN cells at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	211	2423	191	81	922	47
34	148	3038	487	98	1497	854
35	149	2675	935	118	1511	2065
36	180	580	849	68	1562	1114
37	156	215	546	65	477	207
38	307	5521	549	145	2868	1060
183	164	5094	2756	77	2841	1877
184	184	4125	2822	102	2621	1942
245	184	5271	1425	74	2513	887
246	122	5111	2118	81	1768	1673
247	180	4561	732	80	1937	490

Appendix 6.3.5

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SBU-T4 ($CD4^+$ T cells) at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	371	340.5	397.5	162.5	224.5	244.5
34	230.5	209.5	167.5	113.5	132.5	144
35	155.5	323	143	85.5	100	66
36	262	339	217	103.5	315	154
37	240.5	165.5	187.5	139.5	171	165
38	176	160	173.5	168	97	148.5
183	310	201	272	142	125	132
184	247	178	183	111	104	121
245	219	251	240	123	156	157
246	227	229	298	126	121	169
247	238	214	263	138	128	132

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SBU-T4 ($CD4^+$ T cells) at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	371	679	185	162.5	676	68
34	230.5	1030	1018	113.5	280	616
35	155.5	224	587	85.5	141	287
36	262	609	682	103.5	153	440
37	240.5	727.5	1710	139.5	316	185
38	176	205	680	168	367	523
183	310	1179	2082	142	740	1091
184	247	1217	2367	111	556	1212
245	219	979	1763	123	601	1084
246	227	1024	1872	126	478	1077
247	238	1305	1834	138	614	1027

Appendix 6.3.6

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SBU-T8 ($CD8^+$ T cells) at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	205	113.5	225	201.5	199.5	70
34	361	344	141.5	127	128	140.5
35	224.5	243	160.5	199.5	234	161.5
36	180	180.5	372	114	68	167
37	208.5	415	227.5	141	253.5	191.5
38	469	303	368	223.5	205	266
183	166	154	174	109	114	112
184	227	224	239	127	132	160
245	96	171	302	100	117	109
246	178	224	217	108	137	108
247	225	250	232	133	147	160

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SBU-T8 ($CD8^+$ T cells) at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	205	1121	734	201.5	469	403
34	361	880	738	127	234	555
35	224.5	301.5	1301	199.5	65	500
36	180	419	1127	114	259	312
37	208.5	812	652	141	352	378
38	469	606.5	1445.5	223.5	262	680
183	166	416	1208	109	256	446
184	227	482	1214	127	305	581
245	96	654	1003	100	363	652
246	178	601	1172	108	354	665
247	225	709	1152	133	430	622

Appendix 6.3.7

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody 86D (gamma delta T cells) at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	328.5	744	813	197	234	130.5
34	355.5	211	145	69	64	89
35	315.5	275	162.5	420	195.5	199.5
36	83	415	176.5	156.5	298.5	248
37	177.5	138	136	223	224.5	61.5
38	147	323.5	327	180	112	145.5
183	139	201	206	97	158	120
184	152	102	90	97	57	51
245	150	131	153	87	116	86
246	146	162	68	109	108	89
247	108	170	150	71	86	64

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody 86D (gamma delta T cells) at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	328.5	281	682	197	151	440
34	355.5	290	700	69	291	278
35	315.5	184	411	420	252.5	353
36	83	405	161	156.5	135	82
37	177.5	405	407	223	480	211
38	147	273	355	180	226	288
183	139	307	185	97	120	137
184	152	159	173	97	93	137
245	150	192	143	87	144	123
246	146	412	318	109	177	193
247	108	354	341	71	236	139

Appendix 6.3.8

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody OM1 (macrophages) at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	545.5	228.5	732	140.5	213	274.5
34	137.5	150	178.5	139.5	173	148
35	440	241	387	284	204	122
36	179.5	186	174.5	131	103.5	71
37	159.5	116	250.5	208	138	130
38	180	182	262	302.5	128.5	178
183	140	129	178	151	127	167
184	117	143	136	133	163	139
245	125	92	101	106	105	116
246	221	158	161	152	132	114
247	209	169	147	130	242	142

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody OM1 (macrophages) at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	545.5	272	<40	140.5	73.5	<40
34	137.5	175	<40	139.5	71	<40
35	440	245.5	<40	284	178	<40
36	179.5	186	<40	131	68	<40
37	159.5	271	<40	208	120	<40
38	180	359	<40	302.5	167	<40
183	140	32	<40	151	21	<40
184	117	37	<40	133	89	<40
245	125	117	<40	106	132	<40
246	221	129	<40	152	108	<40
247	209	144	<40	130	185	<40

Appendix 6.3.9

Tables indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SW73.2 (MHC class II) at time points following the i/d injection of PBS in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	539	296	154.5	610	200	172
34	234.5	367	291.5	217	209	172.5
35	538.5	421.5	438	296	170	208
36	263	175	300.5	112.5	136	232.5
37	277.5	307	277.5	307	167	145.5
38	225	191	162	161	102	124
183	367	312	442	234	245	265
184	356	356	353	286	256	237
245	526	518	533	351	381	320
246	611	431	400	354	315	370
247	477	490	383	234	350	241

Table indicating the superficial dermal and periadnexal cell count of cells exhibiting positive staining with monoclonal antibody SW73.2 (MHC class II) at time points following the i/d injection of PPD in sensitised sheep

Sheep	Periadnexal count at			Superficial dermal count at		
number	0 hours	24 hours	72 hours	0 hours	24 hours	72 hours
33	539	300	<50	610	193	<50
34	234.5	277	<50	217	397	<50
35	538.5	289	<50	296	263	<50
36	263	612	<50	112.5	254	<50
37	277.5	337	<50	307	272	<50
38	225	454	<50	161	270	<50
183	367	852	<50	234	550	242
184	356	713	<50	286	457	239
245	526	590	<50	351	673	341
246	611	721	<50	354	274	328
247	477	663	<50	234	486	214

APPENDICES TO CHAPTER 7

Appendix 7.3.2

Table indicating the PMN cell count in the superficial and deep dermis for a control biopsy and at four hours post intradermal injection of the indicated solutions

Sheep no.	Superficial dermis					Deep dermis				
	0 hours	rhil8	tnf	zap	saline	0 hours	rhil8	tnf	zap	saline
con RT05	10	416	315	26	3	2	562	685	793	0
con RT135	5	67	564	578	5	2	510	1079	675	58
con RT151	5	100	46	76	2	1	708	524	909	2
con 147	4	114	112	86	4	1	247	519	667	27
con 148	48	162	602	47	85	16	152	866	533	2
con 149	13	61	329	344	5	4	406	670	956	2
mvv 006	2	772	705	110	39	2	562	1370	598	157
mvv 045	3	101	148	44	8	1	370	710	618	2
mvv 065	3	14	278	320	53	2	246	1168	767	248
mvv 075	6	929	361	344	4	2	786	771	657	2
mvv 101	2	1070	889	370	16	2	1082	993	502	1
mvv 115	5	130	5	13	13	2	367	2	471	1

Appendix 7.3.3

Table indicating the $CD4^+$ cell count in the superficial and deep dermis for a control biopsy and at four hours post intradermal injection of the indicated solutions

Sheep no.	Superficial dermis					Deep dermis				
	0 hours	rhil8	tnf	zap	saline	0 hours	rhil8	tnf	zap	saline
con RT05	54	30	40	20	32	77	43	76	38	108
con RT135	40	37	60	13	15	65	92	165	16	15
con RT151	23	35	73	104	47	14	39	140	93	47
con 147	12	17	128	26	11	12	20	78	48	21
con 148	40	21	71	21	12	16	28	24	19	10
con 149	31	36	58	24	74	29	78	86	59	79
mvv 006	20	36	41	58	69	21	66	71	80	90
mvv 045	16	24	27	20	35	8	12	41	33	8
mvv 065	11	62	123	34	50	9	46	187	76	65
mvv 075	77	44	27	12	66	16	38	18	14	44
mvv 101	3	12	60	16	10	5	21	92	19	12
mvv 115	83	31	93	76	28	38	9	37	20	54

Appendix 7.3.4

Table indicating the increase in skin thickness at time points following the i/d injection of PPD in sensitised MVV positive and control sheep

Sheep number	**Increase in skin thickness (mm) at**					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
RT 05	0	1.1	4.00	10.0	13.0	6.0
RT 135	0	1.2	4.67	10.0	12.0	5.0
RT 151	0	1.6	6.67	11.5	13.0	8.0
147	0	1.2	5.33	13.0	12.0	7.0
148	0	1.0	6.67	12.0	13.0	7.0
149	0	0.8	4.67	10.0	13.0	6.0
006	0	0.9	4.33	11.0	12.0	8.0
045	0	0.5	3.67	6.0	9.0	6.0
065	0	0.6	4.00	7.0	7.0	5.0
075	0	0.9	4.00	8.0	9.0	5.0
101	0	0.8	4.00	10.5	9.0	6.0
115	0	0.5	3.33	7.0	8.0	5.0

APPENDICES TO CHAPTER 8

Appendix 8.3.1

Table indicating the mean optical density readings in an anti-PPD antibody ELISA test at a serum dilution of 1 in 40 pre and post vaccination for MVV seropositive and control sheep

Sheep Number	**Mean OD reading at 1 in 40 prior to BCG vaccination**	**Mean OD reading at 1 in 40 following BCG vaccination**
Con 183	0.190	0.067
Con 184	0.134	0.412
Con 245	0.073	0.466
Con 246	0.424	0.770
Con 247	0.146	0.297
MVV 011	0.122	0.251
MVV 025	0.313	0.337
MVV 035	0.433	0.428
MVV 071	0.238	0.396
MVV 076	0.535	0.787
MVV 118	0.234	0.723

Table indicating the optical density readings in an anti PPD antibody ELISA test for serial dilutions of serum taken from sheep 250 pre and post hyperimmunisation

	Serum dilution											
	neat	1 in 25	1 in 50	1 in 100	1 in 200	1 in 400	1 in 800	1 in 1600	1 in 3200	1 in 6400	1 in 12800	1 in 25600
At PM	0.442	0.962	1.249	1.109	1.19	0.919	0.908	0.711	0.739	0.644	0.371	0.212
At PM	0.299	0.878	1.08	1.06	1.092	0.808	0.822	0.632	0.822	0.705	0.42	0.203
At PM	0.559	0.934	0.746	0.847	0.907	0.799	0.92	0.727	0.532	0.531	0.544	0.147
At PM	0.532	0.977	0.973	0.929	0.841	0.681	0.894	0.66	0.484	0.403	0.339	0.12
Post	0.535	0.64	0.941	1.015	1.031	0.707	0.765	0.661	0.791	0.5	0.334	0.203
Post	0.634	0.619	0.752	0.939	0.886	0.61	0.653	0.435	1.004	0.66	0.358	0.178
Post	0.876	0.769	0.718	0.826	0.66	0.702	0.843	0.696	0.525	0.452	0.303	0.16
Post	0.778	0.867	0.661	0.64	0.708	0.755	0.738	0.66	0.518	0.372	0.222	0.16
Pre	0.264	0.063	0.016	-0.096	-0.01	-0.005	-0.001	-0.004	0.014	-0.02	-0.032	-0.043
Pre	0.332	0.106	0.009	-0.057	-0.007	-0.008	-0.004	-0.007	0.048	0.054	-0.024	-0.033
Pre	0.179	-0.087	0.028	0.036	-0.004	0	0.011	-0.008	-0.016	-0.011	-0.011	-0.086
Pre	0.128	-0.054	0.044	0.026	0.008	0.005	-0.004	-0.004	-0.008	0.019	0.001	-0.085

APPENDICES TO CHAPTER 9

Appendix 9.2.4

Nucleotide sequences of the primers and probes used in the PCR assays. The optimum temperature for each assay is listed.

Cytokine	Primers and Probe (listed as 5' to 3' in the order of sense,antisense,probe)	Optimum temperature
ATPase	GCTGACTTGGTCATCTGC CAGGTAGGTTTGAGGGGAT CATCCCCTGCTGGAAGACGGAATT	55°C
POL2	ATAGTAAATGGCATCAAGATGC TCCCGAATTTGTTTCTACCC CATTGGCAAGTGGATT	51°C
IL2-R	ACCTTCCAGGTCACTGCGAGG CTGCGATCTGGTACTCGGTGG GGGCAGACGGTTCACTACCAGTG	61°C
γ-IFN	TGAAATACACAAGCTCCTTC TCACCTTGATGAGTTCATTGA CAAGACATGTTTCAGAAGTTCTTGAACGG	50°C
TNFα	ATGAGCACCAAAAGCATGATCC GAAGAGCGTGGTGGCTCC AGGAGGTGCTCTCCAACAAAGCA	61°C
IL-10	ATGCCACAGGCTGAGAAC GTTCACAGAGAAGCTCAGT ACCTGCTCCACCGCCTTG	54°C

Appendix 9.3.1

Table indicating the increase in skin thickness at time points following the i/d injection of PPD in sensitised MVV positive and control sheep

Sheep number	**Increase in skin thickness (mm) at**					
	2 hours	7 hours	24 hours	48 hours	72 hours	96 hours
064	0	1	4	8	8	5
103	0	1	4	3.5	5.5	3
155	0	0.5	3	9.5	8	4.5
168	0	1.5	4.5	6	6	4
174	0	2.5	6	8.5	6.5	5.5
255	0	1	4	6.5	4	2
51	0	2.5	5	13	11	6
52	0	2	5.5	10	10	6
54		3	5.5	8	9.5	7
55	0	4.5	6	13	11.5	6
56	0	3	4	11	11.5	9.5
57	0	3	6	9	10	9

Appendix 9.3.2

Table indicating the presence of positive bands on autoradiographs of radiolabelled probing of Southern blots of PCR products derived from skin biopsies taken from control sheep

mRNA	Sheep 51			Sheep 52			Sheep 54			Sheep 55			Sheep 56			Sheep 57		
	con	pbs	ppd	con	pbs	ppd	con	pbs	ppd	con	pbs	ppd	con	pbs	ppd	con	pbs	ppd
ATPase	2	3	3	3	3	2	3	1	3	3	3	3	3	3	0	3	3	3
IL-10	1	3	1	1	3	1	3	1	1	3	0	1	0	1	0	3	1	1
TNF	0	0	0	0	0	1	1	0	1	1	0	0	0	1	0	1	1	2
IFN	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
IL2-R	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
POL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

key: 0=no band, 1=weak band, 2=moderate band, 3=strong band

Table indicating the presence of positive bands on autoradiographs of radiolabelled probing of Southern blots of PCR products derived from skin biopsies taken from MVV seropositive sheep

mRNA	Sheep 64			Sheep 103			Sheep 155			Sheep 168			Sheep 174			Sheep 255		
	con	pbs	ppd	con	pbs	ppd	con	pbs	ppd	con	pbs	ppd	con	pbs	ppd	con	pbs	ppd
ATPase	3	0	3	2	3	3	1	1	3	3	3	1	2	3	3	3	3	3
IL-10	2	0	3	1	2	2	0	3	3	3	3	0	0	1	0	1	1	1
TNF	1	0	0	1	1	1	0	1	0	1	1	1	0	0	1	0	0	1
IFN	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
IL2-R	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1
POL 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

key: 0=no band, 1=weak band, 2=moderate band, 3=strong band

PUBLICATIONS

Publications derived directly from this thesis

Pyrah,I.T.G., Watt,N.J.,(1995), Immunohistological study of the cutaneous delayed type hypersensitivity reaction in sheep. Veterinary Immunology and Immunopathology. 48: 299-312.

Pyrah,I.T.G., Watt,N.J.,(1995), Investigation of dermal delayed type hypersensitivity reactions in Maedi-Visna infected sheep. Veterinary Pathology. 32: (5); 573.

Pyrah,I.T.G., Watt,N.J.,(1995), Comparison of the dermal delayed type hypersensitivity reactions in Maedi-Visna infected and control sheep. Accepted for publication in the European Journal of Veterinary Pathology.

Pyrah,I.T.G., Watt,N.J.,(1995), Immunohistological study of the depressed cutaneous DTH response in sheep naturally infected with the ovine lentivirus (Maedi-Visna virus). Revision requested after submission to Clinical and Experimental Immunology.

Publications derived from work associated with this thesis

Collie,D.D.S., Pyrah,I.T.G., Watt,N.J.,(1995), Distribution and quantitation of lung parenchymal contractile tissue in ovine lentivirus-induced lymphoid interstitial pneumonia. Accepted for publication in Laboratory Investigation.

Collie,D.D.S., Pyrah,I.T.G., Watt,N.J.,(1995), Quantitative lung morphometry in sheep: Fixed to physiological lung volume ratios are influenced by delay in fixation. Submitted but not yet accepted for publication.

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Immunohistological study of the cutaneous delayed type hypersensitivity reaction in sheep

Ian T.G. Pyrah *, Neil J. Watt

Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Veterinary Field Station, Easter Bush EH25 9RG, UK

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Abstract

The cutaneous delayed type hypersensitivity (DTH) reaction may be experimentally initiated both as an in-vivo technique for the study of the cell mediated arm of the immune system, and also as an accurate clinical test of the functional capacity of this part of the immune response. This study was performed to fully evaluate the immunohistological characteristics of the normal DTH reaction utilising an ovine model. Six clinically healthy sheep were inoculated with an intradermal *Mycobacterium bovis* vaccine. After 21 days, they were challenged with multiple intradermal injections of a purified protein derivative (PPD) of *M. bovis* in the hairless skin of the medial thigh. Simultaneous contralateral injections of sterile diluent were performed to provide control material. The resulting lesions were measured for increase in skin thickness and biopsied at 2, 7, 24, 48, 72, and 96 h post injection. The biopsies were divided, and stained both histochemically and with monoclonal antibodies directed against lymphocyte subsets, macrophages, and B cells. The DTH reaction was maximal at 72 hours post challenge, and was largely characterised by an initial influx of polymorphonuclear neutrophil (PMN) cells, after which there was an accumulation of $\alpha\beta$ T cells. The number of macrophages within the lesion declined with the progression of the reaction. B cells and $\gamma\delta$ T cells did not appear to play a major role in the response. Fibrin was a marked component of the reaction at later time points.

Keywords: Delayed type hypersensitivity; Sheep; Mycobacterium; Immunology

1. Abbreviations

BCG, Bacillus de Calmette Guerin; DTH, delayed type hypersensitivity; PMN, polymorphonuclear neutrophil; PPD, purified protein derivative.

* Corresponding author: Tel. 0131 650 8812; Fax. 0131 445 5770; E-mail IANP@LAB0.VET.ED.AC.UK.

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2. Introduction

The classical dermal delayed type hypersensitivity (DTH) reaction is an important model for studying the mechanisms of action of the cell mediated immune system, that part of the immune system especially associated with the host response to certain infections such as fungi and mycobacteria, and also the sole in-vivo measure of the competence of the cell-mediated immune response (Otto et al., 1993). There is a surprising paucity of published material that describes the normal immunohistological characteristics of the DTH reaction, with no single study fully evaluating the cellular kinetics of the reaction. Considering the importance of the cell mediated immune response in the control of mycobacterial infections that are becoming increasingly clinically prevalent, and the growing importance of the gross DTH reaction as a clinical measure of cell mediated immune function, especially as a prognostic indicator in human immunodeficiency virus infection (Ahmed and Blose, 1983; Kniker at al., 1984; Bratt et al., 1986; Borleffs et al., 1991; Rosentreich, 1993), this lack of basic information constitutes a serious gap in the understanding of the cell mediated immune response and prevents the scientific evaluation of any clinically apparent alterations in DTH reaction. This study was undertaken in order to fully characterise the DTH response and address this gap in the knowledge of its development, utilising a mycobacterial purified protein derivative (PPD) challenge in a previously immunised sheep as a model.

The results from this study are particularly useful for future investigations of altered DTH responses in all species, with particular relevance to the investigation of the depression in DTH response that has previously been reported to occur in sheep affected with Maedi-Visna virus (Myer et al., 1988), but will also aid in the understanding of the mechanisms of action of the cell-mediated arm of the immune system.

3. Materials and methods

3.1. Animals

Six clinically normal adult sheep, two Finn and four Greyface obtained from a commercial market source, were used in this experiment. A full post-mortem examination was undertaken at the conclusion of the experiment to confirm the absence of intercurrent disease.

3.2. Initiation of the delayed-type hypersensitivity response

The sheep were primed intradermally with five human doses of a *Mycobacterium bovis* Bacillus de Calmette Guerin (BCG) vaccine (Evans Medical Ltd., Langhurst, UK) in the skin of the flank region. After 21 days they were challenged with six separate intradermal injections of 0.1 ml of an *M. bovis* PPD (Central Veterinary Laboratories, Weybridge, UK), equivalent to 0.1 mg of protein or 2500 IU of activity, in the hairless skin of the medial thigh. The site of these injections was standardised via use of a perspex template and skin marker spray, the precise point of injection being marked

circumferentially with permanent black ink. A similar set of six control injections of sterile diluent was made in the contralateral thigh.

3.3. Lesion measurement and biopsy

The increase in skin fold thickness in the lesions was measured using a set of measuring calipers (Camlab Ltd., Cambridge, UK) at 2, 7, 24, 48, 72, and 96 h post injection, with the lesional skin fold thickness being compared with that of adjacent unaffected skin. At the same time point, the measured lesion was anaesthetised using a perilesional 'ring block' of subcutaneous local anaesthetic (Xylocaine 1%, Astra Pharmaceuticals, Kings Langley, UK) and a lesional biopsy taken using an 8 mm disposable punch (Steifel Laboratories Ltd., Woodburn Green, UK). The samples were immediately bisected, with one half being placed in 10% neutral buffered formalin and one half being snap frozen at −70°C in a mixture of dry ice and isopentane. The biopsy sites were closed using surgical staples (Proximate, Ethicon Ltd., Somerville, NJ, USA).

3.4. Specimen staining and evaluation

The formalin fixed specimens were embedded in paraffin, sectioned at 5 μm and stained in a standard manner with haematoxylin and eosin, toluidine blue, carbol chromotrope and Martius scarlet blue. The frozen specimens were sectioned at 6 μm in a cryostat and mounted on tissue adhesive (Biobond, British Biocell International Ltd., Cardiff, UK) coated slides. These sections were stained with monoclonal antibodies directed against the following cell types: CD4 +, CD8 +, $\gamma\delta$ TCR +, B cells and macrophages (Table 1). The monoclonal staining was detected utilising a standard immunoperoxidase detection system (Vectastain Elite, Vector Labs Ltd., Peterborough, UK), and sections were subsequently counterstained with haematoxylin. Microscopical evaluation indicated that the cellular reaction was centred on the adnexal structures. As a result, quantitative evaluation was performed in the specific periadnexal and superficial dermal areas using a squared (10 × 10) eyepiece graticule at a magnification of ×200, in a modification of the method described by Bos et al. (1987). The graticule was

Table 1
Code name, specificity, dilution and reference of the monoclonal antibodies used

Code name	Specificity	Dilution	Reference
SBU-T4	Ovine CD4	1:1000	Maddox et al., 1985
SBU-T8	Ovine CD8	1:1000	Maddox et al., 1985
86D	Ovine $\gamma\delta$ T cell receptor	1:1000	Mackay et al., 1989
OM 1	Ovine CD11c	1:100	Gupta et al., 1993
VPM63	Ovine Fcγ receptor 2	1:100	V.K. Gupta, personal communication, 1994
VPM65	Ovine CD14	1:100	V.K. Gupta, personal communication, 1994
DU2.87	Ovine B cells	1:100	Gupta, 1994

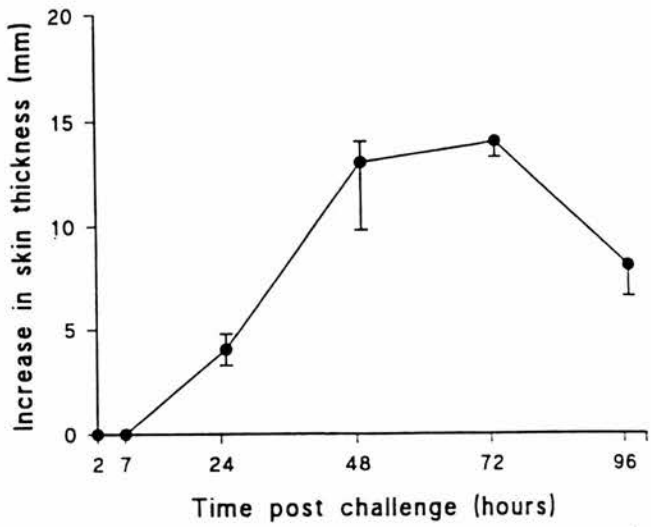

Fig. 1. Increase in the lesional skin thickness versus time (median and interquartile range indicated).

randomly overlaid over an adnexal structure and the cells of interest in ten random squares immediately adjacent to the structure were counted. This was repeated four times, which in most cases was the total number of adnexal structures present. Ten random squares in four adjacent superficial dermal areas were then counted, this again usually constituting the total area of the section. The total number of marked cells in the counted areas was then recorded. A descriptive pathological assessment of the developing lesion was also made.

4. Results

4.1. Skin thickness and histopathological assessment

There was a marked increase in skin thickness in the antigen injected sites, which was maximal at 48–72 h post injection, with no change noted in the control injection sites (Fig. 1).

Histopathologically, the first change in the skin of the biopsies taken from the antigen challenged sites was the presence of marginating polymorphonuclear neutrophils (PMNs) within the dermal blood vessels. These were seen occasionally at the 2 h time, but invariably present at 7 h, where there was an associated migration of these cells into the perivascular spaces. By the 24 h time point there was a universal florid reaction involving largely PMNs, but also some lymphocytes, histiocytes, and fibroblast type cells. This reaction was centred largely on the periadnexal and superficial dermal areas (Figs. 2 and 3). At 48 h post challenge, there had been a reduction in the level of the PMNs and an increase in the numbers of lymphoid type cells, with the marked presence

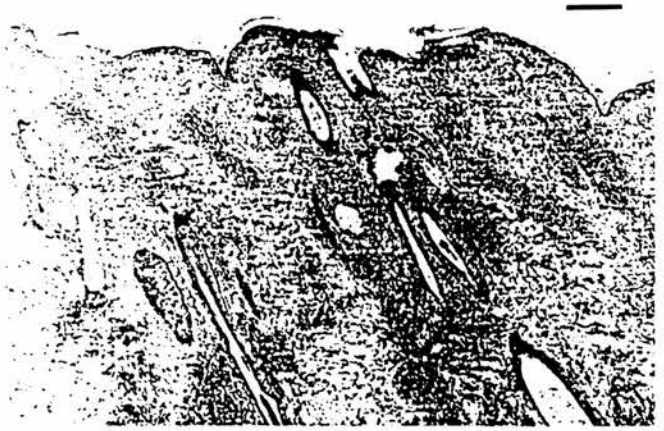

Fig. 2. Low power view of the reaction at 24 h. Note the concentration of cells in the periadnexal areas (H & E, scale bar 200 μm).

of superficial oedema and fibrin. In several samples PMNs were seen within the lumina of hypertrophic sweat glands. The distribution of the cells was more widespread, but was still centred periadnexally and in the superficial dermis (Figs. 4 and 5). In most cases there was an overlying epidermal oedema. The reaction remained largely unchanged at the 72 h time point, before subsiding in cellularity, but not altering in character, at the 96 h time point.

The biopsies taken from the control injected sites exhibited an early mild dermal oedema, with no other significant cellular alteration noticeable.

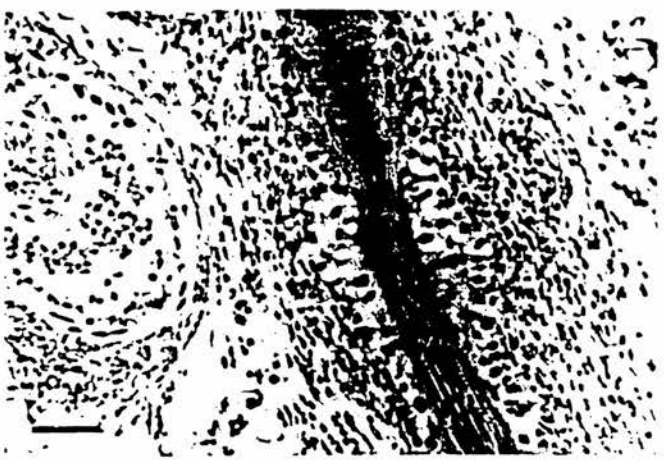

Fig. 3. High power view of the 24 h reaction exhibiting the presence of large numbers of PMNs (H & E, scale bar 50 μm).

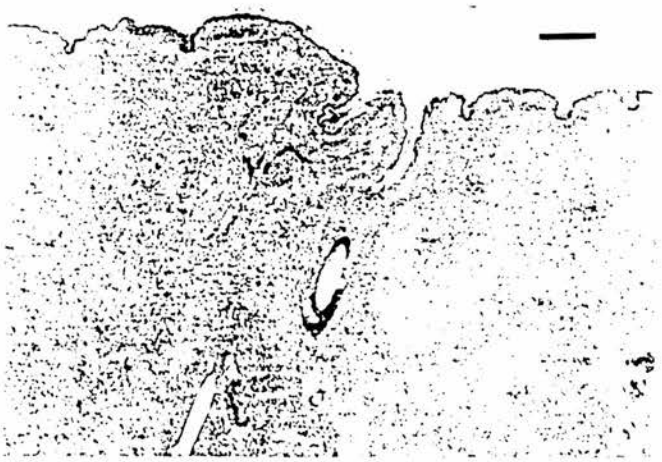

Fig. 4. Low power view of the reaction at 48 h showing the more widespread distribution of cells, although there is still a concentration of cells in the periadnexal areas (H & E, scale bar 200 μm).

4.2. Cell counts

There was a very strong correlation ($P < 0.01$ in all cases) between the cell counts in the periadnexal and dermal compartments, with the densities of cells being proportionally lower in the dermal compartment. The kinetics of the cellular response appeared identical in both areas. The distribution pattern seen probably represents the density of blood vessels in the separate areas, these being the source of the infiltrating cells.

The numbers of PMNs increased dramatically in the early part of the reaction between 7 and 24 h (Fig. 6). The numbers then decreased rapidly to half this level by

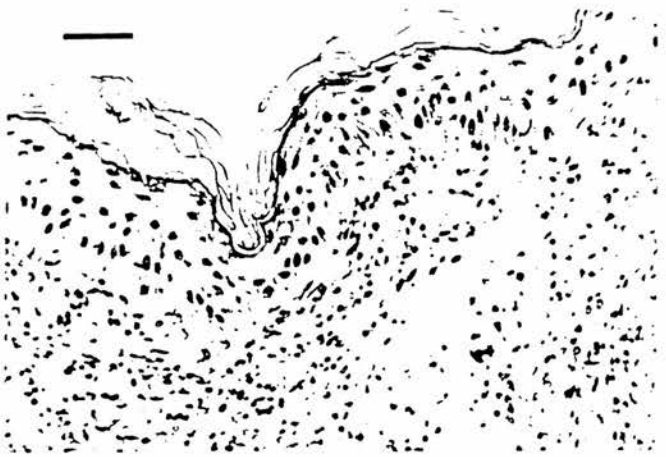

Fig. 5. High power view of the 48 h reaction indicating the decline in PMN numbers with the high levels of mononuclear inflammatory cells (H & E, scale bar 50 μm).

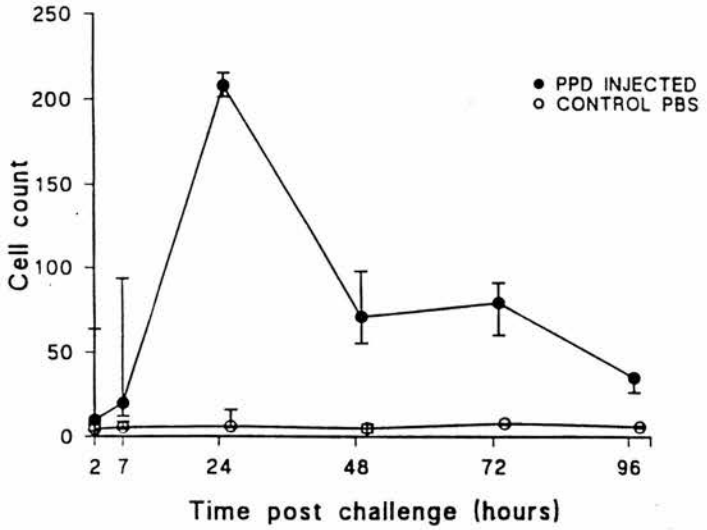

Fig. 6. Periadnexal PMN cell count versus time (median and interquartile range indicated).

48 h and then remained stable to 72 h before returning to almost baseline levels by 96 h. The numbers of PMNs in the control lesions remained unchanged throughout the timecourse of the reaction. The numbers of CD8 + cells in the antigen injected lesion rose slightly at 24 h before reaching a maximal level at 48 h and then remaining relatively constant until the end of the time course (Figs. 7, 8 and 9). Again there was no apparent change in the number of cells in the control lesions.

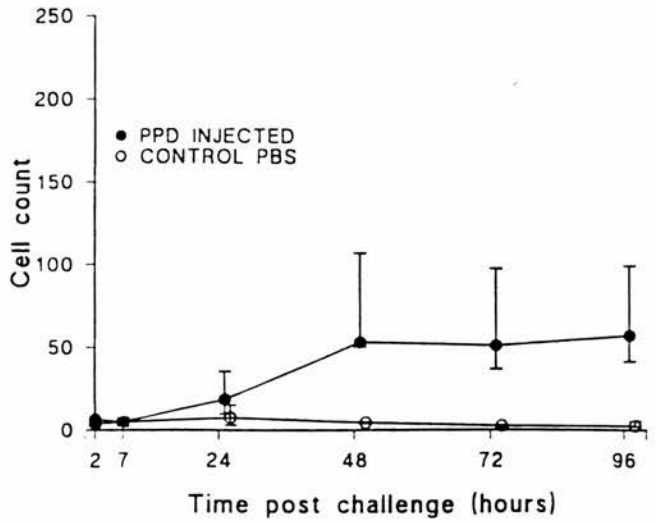

Fig. 7. Periadnexal CD8 cell count versus time (median and interquartile range indicated).

Fig. 8. CD8 + cells in the 24 h reaction (immunoperoxidase, scale bar 100 μm).

Fig. 9. CD8 + cells in the 48 h reaction (immunoperoxidase, scale bar 100 μm).

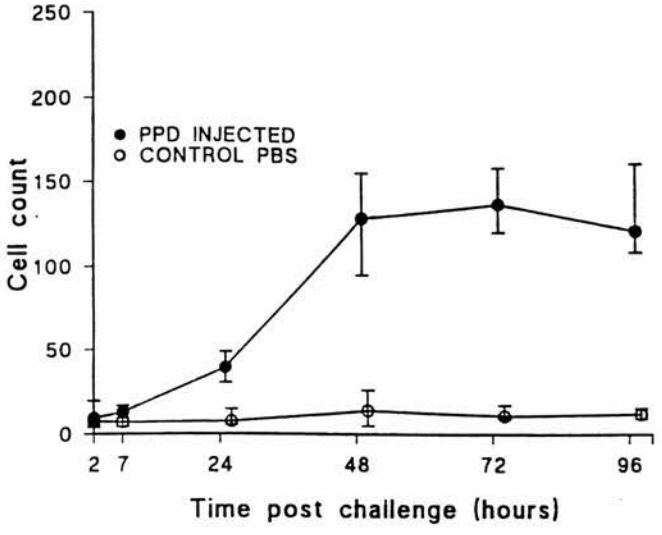

Fig. 10. Periadnexal CD4 cell count versus time (median and interquartile range indicated).

There was a similar result with the CD4 + cells, although the number of CD4 + cells was significantly higher than CD8 + cells. In the antigen challenged skin there was a slight rise at 24 h, with a larger rise to an almost maximal level at 48 h, although the

Fig. 11. CD4 + cells in the 24 h reaction (immunoperoxidase, scale bar 100 μm).

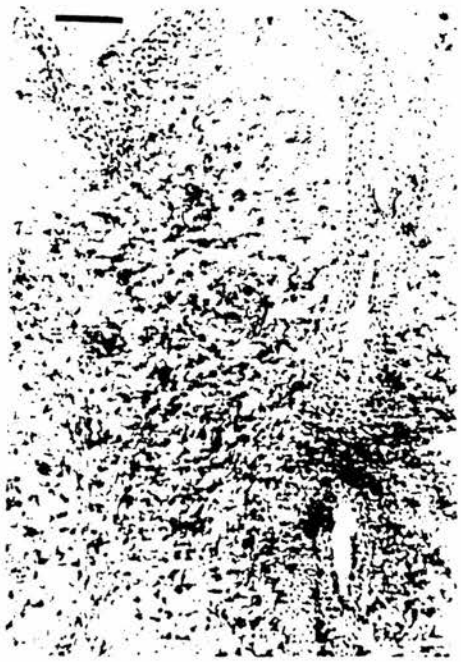

Fig. 12. CD4 + cells in the 48 h reaction (immunoperoxidase, scale bar 100 μm).

highest cell density was recorded at 72 h (Figs. 10, 11 and 12). The control biopsies revealed no significant change in cell counts.

The ratio of CD4 + :CD8 + cells remained relatively constant at approximately 2.5:1 The $\gamma\delta$ T cell positive cells appeared numerically unimportant throughout the reaction, with relatively very low numbers counted when compared with the $\alpha\beta$ T cell positive

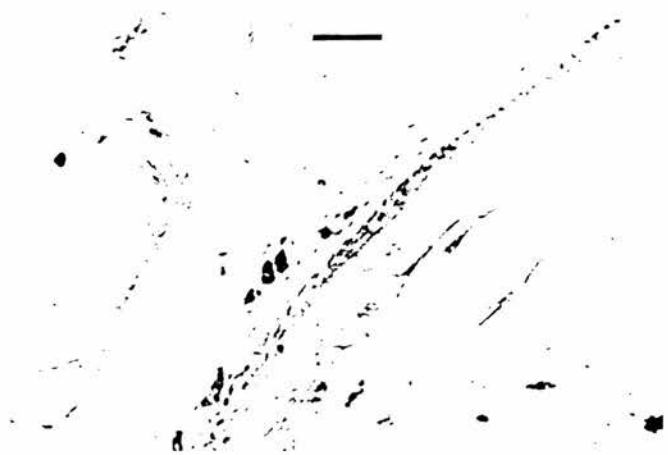

Fig. 13. OM1 stained macrophages at 7 h (immunoperoxidase, scale bar 50 μm).

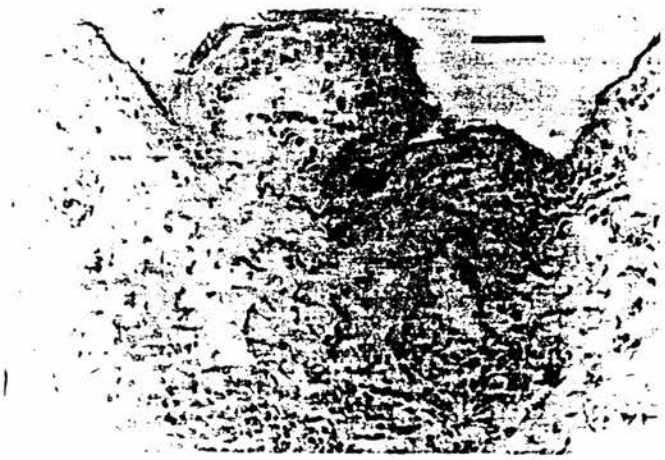

Fig. 14. OM1 stained section at 24 h exhibiting the depressed numbers of macrophages at this time point (immunoperoxidase, scale bar 50 μm).

cells. There appeared, however, to be a slight rise in the numbers of $\gamma\delta$ + T cells throughout the time course of the reaction. There was no significant change in the number of $\gamma\delta$ + T cells in the control lesions.

Macrophages, as recognised by staining with OM1 monoclonal antibody, rapidly decreased in number at the 24 h time point, remaining at low levels throughout the rest of the reaction (Figs. 13 and 14). There was little change noted in the levels of macrophages present in the control sides.

B cells, as defined by staining with monoclonal antibody DU 2.87 (Table 1), were not noted in the reaction in any significant numbers.

Mast cells numbers in all biopsies were similarly relatively low, with no apparent alteration in the antigen reaction.

Fibrin was detected via use of the histochemical stain Martius scarlet blue. It was first noted in the 24 h biopsy, at which point the positively stained material constituted approximately 30% of the section as observed microscopically. The level remained relatively constant to the 72 h time point, before declining slightly at 96 h. Fibrin was present occasionally in very low levels in the control biopsies.

5. Discussion

The most striking finding of this study was the consistent appearance of high numbers of PMNs in the earlier biopsies, with PMN cell counts at 24 h being similar to T lymphocyte counts at 72 h. There have been very few previous histological assessments of the tuberculin reaction, although the early presence of PMNs has been occasionally reported both in humans, notably by Konttinen et al. (1983), and in the cat, by Legendre et al. (1979). The study by Konttinen et al. (1983) suggested that the PMNs

are only present at relatively low levels in the early reaction, and the work by Legendre et al. (1979) did not indicate the kinetics of the PMN appearance and was subsequently challenged by the work of Otto et al. (1993), who considered PMNs to be an uncommon finding in the feline DTH reaction.

The importance of the PMN in the priming and initiation phase of a DTH type reaction was studied by Kudo et al. (1993), who utilised a monoclonal antibody to selectively deplete PMNs from the circulation in a rat/sheep red blood cell model. They discovered that a PMN depletion at both priming and initiation stages of the reaction markedly lowered the eventual size of the gross lesion and the quantitative level of migration of mononuclear cells into the DTH reaction site, indicating that the PMNs have a key role in the initial priming and the subsequent DTH response.

The role of the PMN infiltration in the DTH response is as yet unclear, although their consistent predominance in the early lesion in this study would support the assertion that they have major role in the development of the classic DTH lesion, presumably through their production of proinflammatory mediators.

Another unexpected finding was that the cell counts of macrophages drop to an almost negligible level during the response. Previous studies examining the levels of macrophages fail to utilise monoclonal antibodies, and most studies group macrophages loosely with other mononuclear cells to give a total figure. The absence of macrophage staining in this study may reflect either the loss of the surface marker upon activation/involvement in the lesion, or the trafficking of the macrophages out of the lesion, presumably to the local draining lymph node. The former is unlikely as the authors have undertaken the staining of macrophages active in other sites which remained strongly positive, and the biopsy samples were also stained with two other monoclonal antibodies, VPM63 and VPM65 (Table 1), which produced a similar kinetic pattern of staining. These factors would support the hypothesis that the cells traffic out of the lesion at an early point, and it is most likely that they travel to a local lymph node. A strong macrophage response was noted in a lymph node draining the site of a feline DTH reaction by Legendre et al. (1979), although the trafficking of cells from the reaction to the node was not investigated. In this situation the DTH reaction site constitutes a likely source of these activated macrophages, which could become resident in the local lymph node.

The presence of large numbers of CD4 + and CD8 + T lymphocytes at later time points was as expected and compares with the work of Poulter et al. (1982) and Gibbs et al. (1984). The ratio of these cells is similar to that found in peripheral blood, which suggests that there is no selective mechanism for cellular traffic of these cell types into the lesion.

The low levels of $\gamma\delta$ + T cells and their only slight rise in numbers during the reaction is similar to the findings of Fujita et al. (1993), who considered the $\gamma\delta$ + T cells to be involved not in the initiation, but in the resolution of the contact allergy DTH type lesion in human subjects. The tuberculin DTH model in the sheep is considerably different from this human model, however, in that the ovine has been found to have a much larger proportion of $\gamma\delta$ + T cells in the peripheral circulation (Hein and Mackay, 1991), and the immune response to mycobacterial antigens has been reported to rely considerably on the $\gamma\delta$ + T cell (Janis et al., 1989). With this in mind, a significant

involvement of γδ + T cells was expected, and their almost negligible presence throughout the reaction was somewhat surprising.

The apparent lack of participation of mast cells and basophils agrees with the work of Torii et al. (1993), who indicated that the mast cell was not important in the tuberculin driven DTH reaction, and indicated that the this tuberculin reaction is likely to be initiated and controlled by the macrophage.

This study has outlined the kinetics and relative numerical contribution of the major immune cell populations in the tuberculin driven DTH reaction in the sheep. The significant involvement of the PMN in the early reaction has been discovered, and constitutes an important finding which deserves further detailed study. The low level of macrophages in the later lesion was another unexpected observation, and suggests that these cells may traffic out of the lesion to local lymphoid tissue once the reaction is initiated locally. The low levels of γδ + T cells found in the DTH lesion was surprising, and may be relevant in elucidating the functional significance of this particular lymphocyte subset. The high levels of CD4 + and CD8 + cells in the later reaction is as would be expected in a cell-mediated immune response.

Acknowledgements

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PREDOMINANT ISOTYPE OF SPECIFIC ANTIBODY-SECRETING CELLS IN BRONCHOALVEOLAR LAVAGE FLUID OF CALVES DEPENDS ON THE ROUTE OF INOCULATION USED FOR PRIMING H. HogenEsch, S.E. Torregrosa, D. Boric, H. Park, K. Park, and T.L. Bowersock. Departments of Veterinary Pathobiology and Pharmacy, Purdue University, West Lafayette, IN 47907.

The concentration of IgG1 in secretory fluids of ruminants equals or exceeds that of IgA, in contrast to most monogastric species, in which IgA is the predominant secretory immunoglobulin. The role of IgG1 in protection against microbial pathogens relative to the role of IgA is not understood. It is also not known whether IgG1 is induced by mucosal immunization and whether the IgG1 is locally produced or derived from serum. We have examined whether subcutaneous versus oral priming of calves with ovalbumin (OVA) influences the isotype of anti-OVA antibodies in the bronchoalveolar lavage (BAL) fluid after intrabronchial challenge. Calves were inoculated subcutaneously with 3 mg of OVA in incomplete Freund's adjuvant or orally by inoculation with 5 mg of OVA in encapsulated in alginate microspheres on three consecutive days. Control calves received PBS only. The calves were challenged by intrabronchial inoculation of 5 mg of OVA in PBS on Day 16. Five days later, the presence of anti-OVA antibody-secreting cells (ASC) and antibodies in BAL fluid was measured by ELISPOT assay and ELISA, respectively. Control calves had no detectable antibodies or ASCs. The subcutaneously primed calves had predominantly IgG1-ASCs and IgG1 anti-OVA antibodies, whereas ASCs in orally primed calves were exclusively IgA. A low level of IgA anti-OVA antibodies was detected in the BAL. We conclude from these experiments that after subcutaneous priming IgG1 B cells are induced that home to the lung upon intrabronchial challenge. After oral inoculation, predominantly IgA B cells are induced that home to the lung upon challenge. These studies support the existence of a common mucosal immune system in cattle

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INVESTIGATION OF DERMAL DELAYED-TYPE HYPERSENSITIVITY REACTIONS IN MAEDI-VISNA INFECTED SHEEP. I.T.G.Pyrah and N.J.Watt. Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Roslin, EH25 9RG.

Six sheep naturally infected with Maedi-Visna virus and six matched, uninfected, control sheep were vaccinated intradermally with a Mycobacterium bovis B.C.G vaccine, and subsequently challenged intradermally with a Mycobacterium bovis derived purified protein derivative in order to elicit a delayed type hypersensitivity reaction in the dermis. The lesions produced were measured and biopsied at several time points up to 96 hours post challenge. The biopsies were stained histochemically, and also with monoclonal antibodies directed against various cell markers.

There was a significantly reduced gross reaction in the sheep infected with Maedi-Visna virus, with a marked variation in size of reaction within in this group. A significant reduction in the polymorphonuclear neutrophil and $CD4^+$ cell densities in the early lesion was noted in the Maedi infected group, with a marked variability within the group. The levels of the neutrophils and $CD4^+$ cells in the early lesion correlated significantly with the eventual maximal size of the lesion. The densities of other cell subsets investigated were not significantly different between the two groups.

This work indicates that there are significant reductions in the cell-mediated immunity in sheep chronically infected with Maedi-Visna virus. This reduction is associated with a lowered level of attraction of neutrophils and $CD4^+$ cells to the site of a delayed-type hypersensitivity reaction. The possible explanations for this phenomenon will be discussed.

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CHARACTERIZATION OF CHICKEN AND TURKEY HETEROPHIL ANTIMICROBIAL PEPTIDES. B.G. Harmon, E.W. Evans, F.G. Beach, J.Wunderlich Department of Pathology, College of Veterinary Medicine, The University of Georgia, Athens, GA 30602.

Recently, numerous antimicrobial peptides have been isolated from leukocytes and mucosal surfaces of different vertebrate species. These peptides are believed to be essential for innate disease resistance. We have identified five different antimicrobial peptides in chicken and turkey heterophil granules. These small cationic peptides are structurally similar to beta-defensins found in bovine neutrophils and bovine respiratory mucosal cells. Avian peptides are bacteriocidal for several avian bacterial pathogens including Mycoplasma gallisepticum, Staphylococcus aureus, and Bordetella avium. In vitro microbicidal activity was concentration dependent and the effective peptide concentration for some bacteria was as low as 2 μg/ml. Therefore, these peptides are probably essential for the microbicidal activity of avian heterophils.

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IMMUNOHISTOCHEMICAL IDENTIFICATION OF B AND T LYMPHOCYTES IN FORMALIN-FIXED, PARAFFIN-EMBEDDED FELINE LYMPHOSARCOMAS: RELATION TO FELINE LEUKEMIA VIRUS (FeLV) STATUS, TUMOR SITE, AND PATIENT AGE. M.Jackson, S.Wood, V.Misra, D.Haines. Western College of Veterinary Medicine, 52 Campus Drive, Saskatoon, SK, Canada, S7N 5B4

The lymphocyte phenotype of 70 formalin-fixed, paraffin-embedded feline lymphosarcomas (LSAs) was determined by immunohistochemistry (IHC) using a T cell polyclonal antibody and a B cell monoclonal antibody. Of the 70 LSAs, 67% were T cell, 27% were B cell, and 6% did not stain with either marker.

FeLV antigen was detected by IHC in 54% of LSAs, whereas 74% contained FeLV DNA detectable by polymerase chain reaction (PCR). B cell tumors were FeLV-positive as frequently as T cell tumors using either IHC or PCR. Intestinal tumors were more likely to be B than T cell. The incidence of B and T cell tumors was not different among young (≤3yr), middle-aged (>3yr to ≤8yr), and old (>8yr) cats. However, both B and T cell tumors from old cats were FeLV-positive more often by PCR than by IHC.

B cell tumors in cats are generally considered to be FeLV negative. This study supports a role for FeLV in B as well as T cell tumors in cats of all ages, although LSAs in some older cats may be associated with non-replicating FeLV.